Corpus luteum of the domestic cat and lynx:

structure, regulation and potential mechanisms of its persistence

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Agrar- und Gartenbauwissenschaften

von

Olga Amelkina

Präsident der Humboldt-Universität zu Berlin

Prof. Dr. Jan-Hendrik Olbertz

Dekanin/Dekan der Lebenswissenschaftliche Fakultät

Prof. Dr. Richard Lucius

Gutachterin/Gutachter

1. Prof. Dr. Katarina Jewgenow
2. Dr. Monika Reißmann
3. Prof. Dr. Almuth Einspanier

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Abbreviations

AR	Androgen receptor
BAX	BCL2-associated X protein
BCL2	B-cell CLL/lymphoma 2
CA	*Corpus albicans*
CL	*Corpus luteum*
e/hCG	equine/human chorionic gonadotropin
ESR	Estrogen receptor
FAS	Fas cell surface death receptor (TNF receptor superfamily, member 6)
frCL	Freshly ovulated *corpus luteum*
GPER1	G protein-coupled estrogen receptor 1
P4	Progesterone
perCL	Persistent *corpus luteum*
PGE_2	Prostaglandin E_2
PGF_{2a}	Prostaglandin F_{2a}
PGFM	Prostaglandin F_{2a} metabolite
PGR	Progesterone receptor
PGRMC	Progesterone receptor membrane component
TNF	Tumor necrosis factor
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A
TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B

Zusammenfassung

Der Gelbkörper (*Corpus luteum*, CL) ist eine transitorische Drüse, die im Ovar nach der Ovulation gebildet wird und mit ihrer Progesteron-(P4)-produktion die Trächtigkeit unterstützt. In allen bisher untersuchten Säugetieren endet die sekretorische Aktivität der CL mit Ende der Trächtigkeit oder Laktation, danach werden die CL abgebaut. Eine funktionale Regression der CL, d.h. die Einstellung der P4-Produktion, ist für die Initiation des neuen Ovarialzyklus notwendig. Der Zyklus von Katzenartigen folgt dem gleichen Muster und CL der Hauskatzen regressieren in Funktion und Struktur nach Ende der Laktation. Ganz unterschiedlich verläuft der Zyklus bei einem anderen Mitglied der Familie der Katzenartigen, den Luchsen. Beim Eurasischen Luchs (*Lynx lynx*) und beim Pardelluchs (*Lynx pardinus*) persistieren die CL nach Geburt und Absetzen der Jungtiere für mehr als zwei Jahre und behalten die Fähigkeit P4 zu sekretieren. Diese reproduktionsbiologische Eigenschaft ist einzigartig und ihre funktionelle Bedeutung unklar. Um die Mechanismen der physiologisch persistierenden Gelbköper zu entschlüsseln, ist eine Kenntnis der Grundlagen der Reproduktion bei Katzenartigen essentiell. Die vorgestellte Arbeit wurde initiiert, um die Funktion der ungewöhnlichen persistierenden (per) CL im Luchs zu untersuchen und um mehr über die Fortpflanzung von Katzen insgesamt zu erfahren. Dazu wurden Untersuchungen zu grundlegenden histologischen und hormonellen Aspekten der Lutealphase bei Katzen durchgeführt und der Einfluss des apoptotischen Systems (programmierter Zelltod) sowie die Rezeptivität gegenüber Steroiden bei der Regulation der CL-Funktion angeschaut. Die gewonnenen CL der Hauskatze wurden entsprechend ihrer Histomorphologie (diese Methode wurde in dieser Arbeit etabliert) den Stadien „Anbildung“, „Entwicklung/Aufrechterhaltung“, „frühe Regression“, „späte Regression“ und „*Corpus albicans*“ zugeteilt. Bei den Luchsarten konnten perCL des Eurasischen Luchses (vor der Zuchtsaison) der „frühen Regression“, perCL des Pardelluchs (nach Verpaarung) der „Entwicklung/Aufrechterhaltung“ und frCL (Pardelluchs, nach Verpaarung) der „Anbildung“

zugeteilt werden. In allen Proben wurden das intraluteale P4 und Östrogene bestimmt. Weiterhin wurde die mRNA- und, wenn möglich, die Proteinexpression der protapoptotischen Faktoren BAX, Caspase-3, FAS, Tumor necrosis factor (TNF), TNF Rezeptor 1(TNFRSFA1) und der Überlebensfaktoren (BCL2, TNFRSFB1), sowie des Progesteronrezeptors (PGR), der PGR-Membrankomponente (PGRMC) 1 und 2, des Östrogenrezeptors (ESR) 1 und 2, des G-Protein-gekoppelten Östrogenrezeptors 1 (GPER1) und des Androgenrezeptors (AR) gemessen. Die Ergebnisse weisen darauf hin, dass die Lutealphase der Hauskatze durch FAS, Caspase-3 und die TNF Rezeptoren 1 und 2 reguliert sein könnte. Steroide könnten über ihre Rezeptoren PGR, PGRMC1 und PGRMC2, ESR1 und AR wirken. Die physiologische Persistenz der Gelbkörper beim Luchs könnte über BCL2, FAS, TNFRSFB1, PGRMC1, PGRMC2, ESR1, GPER1 und AR vermittelt werden. Hier scheinen BCL2 und TNFRSFB1 eine Rolle für das strukturelle Überleben der perCL zu spielen, während die gelisteten Steoridrezeptoren die luteotropen Signale von Progesteron, Östrogenen und Androgenen vermitteln. Diese Steroide sind wahrscheinlich in das Aufwecken (revival) der regressierenden CL (Eurasischen Luchs) zu aktiven CL (Pardelluchs, nach der Verpaarung) involviert. Die vorliegende Arbeit zeigt wichtige Unterschiede in der CL Funktion und Regulation zwischen der Hauskatze und den Luchsen auf und unterstreicht die Notwendigkeit des artspezifischen Vorgehens bei Studien zur Reproduktionsbiologie. Grundlagenkenntnisse zur felinen Fortpflanzung sind wichtig für die Entwicklung von Methoden der Assistierten Reproduktion in Arterhaltungsprogrammen von Katzenartigen. Weiterhin ermöglicht das Verständnis der Funktion von perCL die Entwicklung von Maßnahmen zur Zyklussteuerung und Ovulationsinduktion bei Luchsen und trägt somit zum Erfolg des Zuchtprogrammes des hochbedrohten Pardelluchses bei.

Summary

Corpus luteum (CL) is a transitory gland which forms in the ovary after ovulation and supports the pregnancy with its production of progesterone (P4). In all mammals studied so far, the CL loses its secretory activity by the end of pregnancy or lactation and eventually regresses from the ovary. The functional regression of the CL, i.e., decreased P4 production, is critical for the initiation of a new ovarian cycle. The luteal cycle of felids follows the same pattern, and CL of the domestic cat functionally and structurally regress after lactation. However, the story is different for another member of the *Felidae* family, the lynx. In the Eurasian (*Lynx lynx*) and Iberian (*Lynx pardinus*) lynx, CL persist after parturition, weaning and for up to two years, still retaining their ability to secrete P4. To date, this reproductive characteristic is unique and its function in lynx species is unclear. Broad knowledge on basic feline reproduction is required to unravel the potential mechanisms of physiological CL persistence. Current work was initiated to understand the control of unusual persistent (per) CL in lynx and to learn more about feline reproduction in general. For this, studies on the basic histological and endocrinological aspects of the feline luteal phase, as well as potential involvement of systems of apoptosis (programmed cell death) and steroid receptivity in the CL regulation were performed. Collected CL from domestic cats were classified based on their histomorphology (method established in this study) as stages of formation, development/maintenance, early regression, late regression and *corpus albicans*. For two lynx species, CL were staged as early regression (perCL, Eurasian lynx, before mating), development/maintenance (perCL, Iberian lynx, post mating) and formation (frCL, Iberian lynx, post mating). In all samples, intraluteal P4 and estrogens were measured. Moreover, mRNA and where possible protein levels were determined for pro-apoptotic BAX, caspase-3, FAS, tumor necrosis factor (TNF), TNF receptor 1 (TNFRSFA1), and pro-survival BCL2 and TNFRSFB1, as well as progesterone receptor (PGR), PGR membrane components (PGRMC) 1 and 2, estrogen receptors (ESR) 1 and 2, G protein-coupled estrogen receptor 1

(GPER1) and androgen receptor (AR). The results suggest that the luteal phase of the domestic cat is potentially regulated by caspase-3, FAS, TNFRSF1A, TNFRSF1B, and by actions of steroids *via* PGR, PGRMC1, PGRMC2, ESR1 and AR. Physiological persistence of Iberian lynx CL might be mediated by BCL2, FAS, TNFRSFB1, PGRMC1, PGRMC2, ESR1, GPER1 and AR. Here, BCL2 and TNFRSFB1 might play a role in structural survival of perCL, while listed steroid receptors might transduce the luteotropic signals of progesterone, estrogens and androgens. These steroids can thus play a role in the revival of the regressing perCL (Eurasian lynx, before mating) and their transition into maintained perCL (Iberian lynx, post-mating). Current work indicates profound differences between the CL function and regulation in domestic cats and lynx, and promotes a highly species-specific approach in reproduction studies. Fundamental knowledge on the feline reproduction is critical for the development of assisted reproductive techniques in conservation of wild felids. Moreover, understanding the control mechanisms of lynx perCL would enable supportive manipulations of the ovarian cycle and contribute greatly to the breeding programs for the endangered Iberian lynx.

1 INTRODUCTION

1.1 Mammalian corpus luteum – a major source of progesterone

The gland *corpus luteum* (CL; translates 'yellow body') acquired its name in 1681 by Marcello Malpighi, as he introduced this term in a letter to Jacobo Spon. The first clear description and drawings of the CL were carried out by Regnier de Graaf, who observed the presence of 'globular bodies' in the rabbit ovary after coitus. These 'globular bodies' remained in the ovary until after parturition and corresponded to the number of fetuses *in utero*. Subsequently in 1898, Prenant studied the histology of the CL and proposed a secretory activity of this tissue: "there can be no doubt… it (the *corpus luteum*) acts as a gland, and as a gland of internal secretion…". The hypothesis that the CL is a gland of internal secretion related to pregnancy was brought forth by Gustav Born, and in 1901 two independent experiments by Ludwig Fraenkel in Germany and Vilhelm Magnus in Norway showed that indeed the removal of CL in mated rabbits results in pregnancy failure. Later, Fraenkel supported this observation with follow-up experiments on about 400 rabbits, stating that "thus by the power of large numbers my thesis is proven: the ovary, in particular the *corpus luteum*, regulates the implantation and initial development of the egg". Corner and Allen could prepare a relatively pure alcoholic extract of sow CL and demonstrated that this extract maintained pregnancy in ovariectomized rabbits. In 1934, four independent laboratories reported the isolation of the pure crystalline hormone; the same year Slotta *et al* named this compound progesterone (P4) and suggested a structural formula, and it was synthesized by Butenandt and Westphal. The early history of CL discovery in this paragraph is compiled from reviews of Allen and Meyer (1935), Simmer (1970) and Diaz et al. (2002).

It is known now that the CL plays a critical role in the pregnancy maintenance and regulation of the estrous cycle in mammals, mainly by being a transient major source of P4 (Niswender et al. 2000). Once this gland is formed in the ovary after ovulation, it acquires an extensive blood

supply and undergoes rapid hypertrophy. For example, bovine CL can increase in weight from on average 640 mg on day 3 after ovulation to 5.1 g on day 14 (Fields and Fields 1996). Interestingly, during its growth and maintenance, the rate of blood flow to the CL exceeds that of other tissues, and due to its high metabolic demands, the CL consumes two to six times more oxygen per unit of weight than does liver, kidney, or heart (Swann and Bruce 1987). By the end of its life span, the CL regresses from the ovary, leaving a non-functional scar *corpus albicans* (CA) that is eventually reabsorbed. The ovary thus doesn't usually contain the CL until the next ovulation.

Variations in reproductive patterns are extremely high across mammalian species, contributing in particular to their adaptation strategies for likely more efficient reproduction under different environments. There indeed might be no area of mammalian physiology where interspecies variation is so prominent as in the endocrine regulation of the ovarian cycle (McCracken et al. 1999). In this regard, differences in the life cycle and P4 production of the CL contribute greatly to species-specific reproduction patterns. For instance, in several species, e.g., dogs, rats, mice, pigs, goats, the CL is the sole source of P4 throughout the pregnancy, and ovariectomy mainly results in abortion (Hoffmann et al. 1992, Kowalewski et al. 2011, McCracken et al. 1999). The function of P4 main production switches to placenta (luteoplacental shift) at mid or late pregnancy in sheep, horses and primates (Al-Gubory et al. 1999, Allen 2001a, Niswender et al. 2000, Weems et al. 1992). Here, ovariectomy after certain point of pregnancy doesn't change the normal development and delivery of a fetus. Apart from the luteoplacental shift, horses also exhibit the formation of accessory CL during pregnancy (Allen 2001b). These CL are formed from non-ruptured follicles and, therefore, don't correlate to the number of ovulated oocytes. Similar strategy of accessory CL can be observed in the mammal with one of the longest existing pregnancies – the elephant. In this species, accessory CL are hypothesized to be a necessary additional source of progestagens, supporting the pregnancy span of around 600 days (Hildebrandt et al. 2011). These are only some examples

of interspecies variations regarding the CL life cycle. The case of local P4 contribution of placenta in domestic cats will be introduced below (Section 1.5), as well as the peculiar strategy of lynx to maintain the CL outside their typical luteal life span (Section 1.6).

Progesterone exhibits pleiotropic physiological effects in multiple sites of the organism, and the target cells containing its receptor have been discovered in human ovary, uterus, testis, brain, pancreas, bone, mammary gland and urinary tract (Gellersen et al. 2009, Kowalik et al. 2013). In the female reproduction tract, P4 is known to support the environment for the early embryonic development, e.g., by inducing quiescence of the myometrium, acting on endometrium as a differentiation factor and playing a role in placenta development (Peluso 2006). Among the diversity of P4 effects, the one of particular interest is the ability of P4 to inhibit follicular development, thus partly governing the length of ovarian cycles (Buffler and Roser 1974, Kim and Greenwald 1987, Peluso 2006, Setty and Mills 1987, Stouffer 2003). During the follicular phase, the production of P4 is usually low, and estradiol acts on hypothalamic-pituitary-axis to stimulate the release of luteinizing hormone (LH), bringing follicular development to the point of ovulation. After ovulation, increased levels of P4 act on both hypothalamus and pituitary, and restrict secretion of LH, block surges of gonadotropin-releasing hormone and follicular-stimulating hormone (Niswender et al. 2000). This action of P4 on gonadotropins, however, seems to be dependent on the overall endocrine environment, and in some cases, P4 can facilitate surges of gonadotropins induced by estradiol (Krey et al. 1993). Another P4 effect that will be further discussed in this work is the autocrine regulation of its own secretion from luteal cells.

1.2 Formation and structural composition of the corpus luteum

With the preovulatory LH surge, LH receptors in follicular cells are activated, and the program for terminal differentiation of follicular cells into non-dividing P4-producing luteal cells (termed luteinization) is initiated (Stocco et al. 2007). This reprogramming of follicular

cells requires the exit from the cell cycle, and luteal cells are usually found arrested at the G_0/G_1 phase (Green et al. 2000). The LH surge alters the steroidogenic pathway in cells towards the primarily P4 production, and luteinized cells begin to express a new set of molecules required for creating a different hormonal environment for the CL survival. In some species, e.g., humans, pigs and rats, the CL retains ability to produce estrogens for various periods of time (Richards and Hedin 1988, Wuttke et al. 1997, Wuttke et al. 1998). Luteal cells require provision of cholesterol as a substrate for steroidogenesis, which is transported to cells in the form of lipoproteins. Once free cholesterol enters the cytosol of the cell, it can be either used immediately for steroidogenesis or formation of cell membranes, or it can be stored in as cholesterol esters (Johnson et al. 1997). These cholesterol esters can form lipid droplets that are long known as morphological characteristic of steroidogenic cell types.

The fully formed CL consists of steroidogenic luteal cells, derived from granulosa and theca cells, and non-steroidogenic cells of immune and vascular systems and fibroblasts. Such heterogeneous composition of the gland is the result of intensive cell migration after the breakdown of the follicular basal membrane and changes in extracellular matrix. These structural alterations promote neovascularization of the CL, which include development of a dense capillary network for efficient supply of nutrients, hormones and lipoproteins to the CL, as well as for rapid output of P4 from luteal cells (Reynolds et al. 2000). During the CL growth, most of the dividing cells (around 85% in primate CL) are endothelial cells, who recruit smooth muscle cells and pericytes for stabilization of vessels and control of their function (Tamanini and De Ambrogi 2004). Major contribution to the CL rapid growth comes from the hypertrophy of luteal cells, which can increase twice in their diameter in the domestic cat (Arikan et al. 2009). In addition, luteal cells of bovine CL have been shown to exhibit proliferative activity during development stages (Yoshioka et al. 2013).

Immune cells in the CL include primarily T-lymphocytes, macrophages and neutrophils (Bukulmez and Arici 2000). Their role has been discussed during CL formation and regression

as being directly involved in the process of cell death; in rodents and humans, this process can be partly inhibited by P4 action on lymphocyte proliferation and function (Pate and Landis Keyes 2001, Siiteri and Stites 1982). Additionally, macrophages were shown to play a critical role in the development of vascular network in the CL of mice, by providing trophic support for its formation, and thus contributing to the CL integrity and function (Care et al. 2013).

1.3 Apoptosis in the luteal phase

At the end of its lifespan, the CL undergoes the loss of P4 secretory capacity and a subsequent involution from the ovary, termed functional and structural luteolysis respectively. The event of structural luteolysis has been shown to occur mainly *via* apoptosis (McCracken et al. 1999). Apoptosis is derived from Greek “apo” meaning leaf and “ptosis” meaning to drop, and is a term for the programmed cell death. This process is evolutionary conserved among species and is divided into extrinsic and intrinsic pathways. The intrinsic signaling cascade is generally activated by apoptotic stimuli within a cell in response to stress, radiation, certain drugs, or withdrawal of growth factors. During the signal transduction, the permeability of mitochondria is changed *via* alterations in the ratio of anti-apoptotic, e.g., B-cell CLL/lymphoma 2 (BCL), BCL2-like 1, Myeloid cell leukemia 1, to pro-apoptotic, e.g., BCL2-associated X protein (BAX), BCL2-associated agonist of cell death, BCL-2-antagonist/killer, BCL2 binding component 3, members of the BCL2 family (Borner 2003). The extrinsic signaling cascade is activated by extracellular signals, e.g., Fas ligand and tumor necrosis factor (TNF), that interact with cell surface receptors, e.g., FAS and TNF receptor superfamily (TNFRS) members 1 and 2 (Nagata 1997).

Both intrinsic and extrinsic pathways lead to activation of the caspase family, i.e., caspase-9 and -8 respectively, and subsequently the final executioners caspase-3, -6 and -7. These effector caspases cleave a variety of intracellular polypeptides that are critical for the cell survival, including major structural elements of the cytoplasm and components of the DNA repair

machinery (Earnshaw et al. 1999). The importance of caspase-3 protein in the CL regression has been shown on the caspase-3 null mice, where CL exhibited low rates of apoptosis and delay in their involution (Carambula et al. 2002). These CL, however, could finally involute, indicating that caspase-3 is not the only factor leading to cell death in the CL.

Apart from apoptosis of luteal cells, the CL regression has also been linked with necrosis of luteal steroidogenic cells due to the apoptosis of luteal endothelial cells in rats (Gaytan et al. 2002). In primates, regression of the CL was associated with autophagocytosis, another form of programmed cell death characterized by formation of cytoplasmic vacuoles (Fraser et al. 1999). Overall, it is possible that the type of programmed cell death during luteal regression, i.e., apoptosis, necrosis, autophagocytosis, may differ depending on the luteolytic trigger, physiological and pathological conditions, as well as the species (Davis and Rueda 2002).

1.4 Steroid action in the corpus luteum

The mentioned above (Section 1.1) ability of P4 to regulate its own secretion from the ovary was first discussed by Rothchild (Rothchild 1996). Further studies of this concept were complicated due to the absence of nuclear progesterone receptors (PGR) in the rat luteal cells (Park-Sarge et al. 1995). It was later discovered, however, that rat CL contain a number of membrane P4 receptors that can transduce P4 biological effects (Cai and Stocco 2005). Progesterone, therefore, can act through its nuclear receptors, which are currently recognized as three isoforms PGRA, PGRB and PGRC, and membrane receptors, including multiple membrane progesterone receptors (mPRs) and PGR membrane components (PGRMC) 1 and 2 (Kowalik et al. 2013, Peluso 2006). Similarly, estrogen also can transduce its effect *via* nuclear estrogen receptor (ESR) 1 and 2, potential membrane receptors mERs and G protein-coupled estrogen receptor 1 (GPER1; Bottner et al. 2014, Maggiolini and Picard 2010, Rosenfeld et al. 2001, Soltysik and Czekaj 2013). Another steroid complex that acts in luteal cells are androgens and their nuclear receptors AR (Slomczynska et al. 2006, Szoltys et al. 2007).

An extensive amount of studies showed that steroid hormones indeed are able to transduce a variety of their actions in the CL *via* their nuclear and/or membrane receptors. Such actions include P4 stimulation of its own secretion from luteal cells and protection of the CL from the regression (Engmann et al. 2006, Goyeneche et al. 2003, Luciano et al. 2011, Peluso 2006, Rekawiecki et al. 2008). In rats and rabbits, estrogens directly regulate CL formation and maintenance (Rosenfeld et al. 2001) and have been long known to exhibit luteotropic function (Townson et al. 1996). In the CL of mice, estrogens have been recently shown to stimulate P4 synthesis *via* GPER1 (Liu et al. 2015). On the contrary, in primates, estrogens act luteolytic (Duffy et al. 2000) and are proposed to be involved in relocation of the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) receptors within luteal cells and thus induction of $PGF_{2\alpha}$ luteolysis (Kim et al. 2015). In cows, exogenous estrogen administration initiated luteolysis, and it is argued that estrogens from growing follicles can regulate the length of bovine luteal phase (Salfen et al. 1999). Finally, androgens have been shown to stimulate P4 secretion in rat CL directly and without their prior conversion to estrogens, and delay DNA fragmentation in postpartum CL (Carrizo et al. 1994, Goyeneche et al. 2002, Takiguchi et al. 2000). It is worth mentioning that steroid hormones can also elicit rapid but receptor-independent effects by affecting physiochemical membrane properties (Gellersen et al. 2009).

1.5 Ovarian cycle of felids and the domestic cat in particular

The feline estrous cycle is typically divided into four phases: proestrus, estrus, diestrus and anestrus (or interestrus). In the domestic cat, proestrus may last for only one to two days and is characterized by follicle development, rise in serum estradiol, and occasional affection behavior of queens excluding mating permission (Shille et al. 1979). This short and sometimes very subtle stage often remains undetected. During estrus, felids are receptive to mating and exhibit such behaviors as vocalization, lordosis, rolling, rubbing and foot treading. This stage is characterized by the peaks of follicular activity and estradiol secretion, and its length differs

between felids, being on average seven days for the domestic cat. If the queen doesn't ovulate during estrus, she will enter interestrus – a non-luteal period of about one week (for polyestrous domestic cat) till the onset of a next proestrus (Bristol-Gould and Woodruff 2006). In the event of a successful ovulation during estrus, the queen enters diestrus – a luteal phase that is characterized by the formation of one or more CL that produce P4 for various lengths of time. The CL can either belong to pregnancy, which lasts for approximately 65 days in the domestic cat (Tsutsui and Stabenfeldt 1993), or non-pregnant luteal phase (also known as pseudopregnancy), which lasts in felids one-half to two-thirds of the pregnancy span (approximately 40 days in the domestic cat; Wildt et al. 1981). Finally, anestrus is the period in between waves of follicular development, when serum estradiol and progesterone are at baseline levels, and queens are sexually inactive.

Felids have been historically defined as induced ovulators, requiring a mating stimulus for ovulation. It is now known, however, that feline species exhibit a variety of ovulatory patterns, ranging from almost exclusively induced to the combinations of induced and spontaneous. For instance, tiger (*Panthera tigris*), puma (*Felis concolor*), snow leopard (*Panthera uncia*), cheetah (*Acinonyx jubatus*), tigrina (*Leopardus tigrinus*) and ocelot (*Leopardus pardalis*) never or rarely ovulate spontaneously, while the incidents of spontaneous ovulation occur occasionally in the lion (*Panthera leo*), leopard (*Panther pardus*), Pallas' cat (*Otocolobus manul*), fishing cat (*Prionailurus viverrinus*), and are regular for the clouded leopard (*Neofelis nebulosa*), margay (*Leopardus wiedii*) and domestic cat (Brown 2011).

Seasonal and mono/polyestrous patterns are additional variation factors in the feline reproduction. The domestic cat is seasonally polyestrous and, therefore, can mate several times per year, but its ovarian activity is usually restricted to long-day periods (Hurni 1981). Many non-domestic felids also exhibit seasonal reproduction, including tiger, clouded leopard, Pallas' cat and snow leopard. Other felids like the lion, leopard, puma, margay, ocelot, tigrina, jaguar (*Panthera onca*) and fishing cat are not seasonal (Brown 2006).

INTRODUCTION

The typical ovarian cycle of the domestic cat is depicted in the Figure 1. On the mating day, the peak estradiol level (around 60 pg/ml) is followed by a drop (8 – 12 pg/ml) during subsequent five days (Verhage et al. 1976). In pregnancy, estradiol levels remain low until about days 58 – 62 and then rise prior parturition; in pseudopregnancy, or the non-pregnant luteal phase, estradiol shows similar pattern during the first 40 days, but then deviates with individual variations of occasional rise. During anovulatory cycle, number and magnitude of estradiol surges varies with mean peak of around 60 pg/ml (Verhage et al. 1976).

Serum P4 is undetectable or basal during the first one to three days after mating (0.5 – 1 ng/ml) and begins to rise on day four, exceeding 20 ng/ml by day six and peaking at around 40 ng/ml (pregnancy) or 24 ng/ml (pseudopregnancy) on days 14 – 21 (Verhage et al. 1976, Wildt et al. 1981). In pregnancy, serum P4 gradually declines after its peak till day 50 (around 12 ng/ml) and through days 63 – 65 (4 – 5 ng/ml) until reaching nadir concentrations (< 1 ng/ml) immediately after parturition. In pseudopregnancy, serum P4 levels decline to around 4 ng/ml by day 40 and can reach nadir concentrations depending on the individual, e.g., by day 42 (Wildt et al. 1981) or after day 62 (Verhage et al. 1976). During anovulatory cycle, mean P4 levels are constantly below 1 ng/ml.

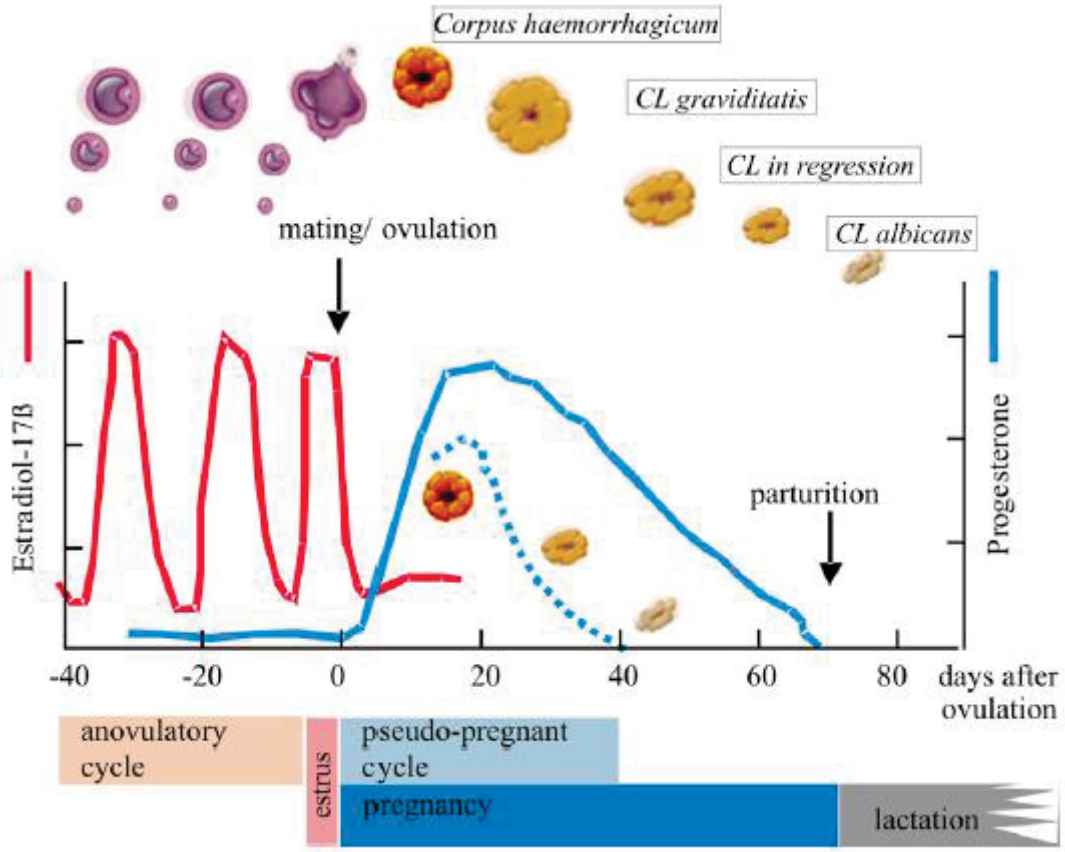

Figure 1. Generalized ovarian cycle of the domestic cat with serum levels of progesterone and estradiol-17β. Presented are stages of the CL throughout its life span (Jewgenow et al. 2014).

The difference in serum P4 peaks between pregnancy and pseudopregnancy raised a question concerning the possible cause. Some studies discussed the potential contribution of placenta to these differences (Gudermuth et al. 1997) and even a possible luteoplacental shift, as suggested by detected activity of steroidogenic enzyme in placenta (Malassine and Ferre 1979). However, experiments with ovariectomy in cats on different stages of pregnancy resulted in a conclusion that the ovary is the only source of peripheral P4 during pregnancy (Tsutsui et al. 2009, Verstegen et al. 1993). Subsequently, it has been revealed that placenta is indeed capable of P4 biosynthesis, however, its contribution must be local and does not influence serum P4 profile (Braun et al. 2012, Siemieniuch et al. 2012). Therefore, in the domestic cat ovarian cycle, the CL is the major source of P4 throughout pregnancy with placenta being an additional but not

sufficient site of P4 biosynthesis. Apart from P4 synthesis, feline CL has a steroidogenic capacity to produce estrogens and androgens (Zschockelt et al. 2015, Zschockelt et al. 2014)

1.6 Ovarian cycle of the lynx

The *Lynx* genus consists of four species that inhabit different parts of the world: the Eurasian lynx (*Lynx lynx*) in the broad range of Europe and Asia, the Canada lynx (*Lynx canadensis*) and the bobcat (*Lynx rufus*) in North America, and the Iberian lynx (*Lynx pardinus*) in the Iberian Peninsula. Eurasian, Canada and Iberian lynx are seasonally monoestrous with a reproduction activity evident in January – April, February – April and January – February respectively (Fanson et al. 2010, Kvam 1991, Palomares et al. 2005, Poole 2003). The bobcat is seasonally polyestrous and has been recorded to ovulate up to three times during the breeding season, the length of which is highly dependent on the latitude and may last from January to July (Nova Scotia; Parker and Smith 1983), February to June (Wyoming; Crowe 1975) or February to March (Mississippi; Stys and Leopold 1993). Although the bobcat is defined as seasonal breeder, there are occasional reports on pregnant females in late autumn (Ohio; personal communication with Ohio Division of Wildlife). All lynx are originally defined as induced ovulators, however, cases of spontaneous ovulation are also evident. In this regard, it is possible that if a monoestrous Eurasian lynx does not mate in time, the ovulation still occurs and results in an infertile luteal cycle (Painer et al. 2014b). Moreover, the bobcat has been shown to primarily ovulate spontaneously (Stys and Leopold 1993).

The reproductive cycle of the lynx is extremely unusual and differs not only from other members of the *Felidae* family, but from any other mammalian species studied so far (Figure 2). Formed after ovulation CL do not regress after parturition and even weaning, but are still present in the ovary by the next estrus and may persist for a period of at least two years (Eurasian lynx; Painer et al. 2014b). Moreover, such persistent CL (perCL) do not lose their functional activity and continue to produce P4, as evidenced by serum (Iberian and Eurasian lynx; Goritz

et al. 2009, Painer et al. 2014b), metabolite (Iberian, Eurasian and Canada lynx; Dehnhard et al. 2008, Fanson et al. 2010, Jewgenow et al. 2009) and intraluteal evaluations (Eurasian lynx; Carnaby et al. 2012). In the bobcat, CL are thought to persist throughout the animal life span (Crowe 1975, Duke 1949), however, the dynamics of their regression and a question of perCL functional activity has not been investigated fully yet.

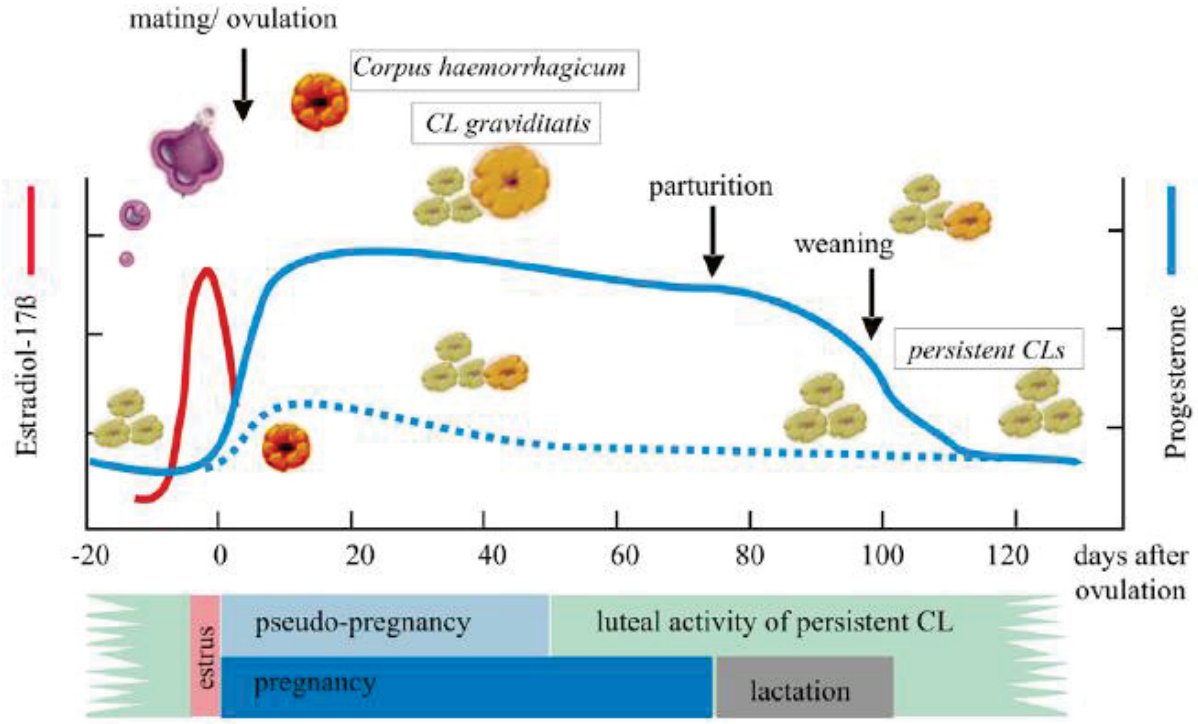

Figure 2. Generalized ovarian cycle of the Eurasian lynx with serum levels of progesterone and estradiol-17β. Presented are stages of the CL throughout its life span (Jewgenow et al. 2014).

Thus, at least three lynx species (Eurasian, Iberian and Canada lynx) do not follow the typical for felids ovarian cycle described above (Section 1.7). Instead of entering anestrus after pregnant or non-pregnant luteal phase, these animals remain in diestrus, termed prolonged diestrus (Painer et al. 2014b), with functional perCL throughout the year. These perCL are hypothesized to secure monoestrous cycle by suppressing ovarian activity with constant P4 production. There is a big question on how do lynx species enter the subsequent estrus and whether it requires a transit down regulation of the CL P4 production. If such down regulation is present, what are the mechanisms that initiate and implement this process? Is it possible to

stimulate functional and/or structural regression, and induce ovulation *via* standard protocols of artificial reproductive techniques used in felids? First experiments on the Eurasian and Iberian lynx for the induction of the CL structural regression and subsequent ovulation by commonly luteolytic $PGF_{2\alpha}$ proved to be unsuccessful (Painer et al. 2014a) and indicate a great need of profound studies on the basic reproduction in lynx species.

Broad knowledge on the lynx reproduction would contribute to the development of assisted reproduction techniques for the endangered Iberian lynx (Comizzoli et al. 2009, Palomares et al. 2011, Swanson 2003). This species has only recently been listed as critically endangered by the International Union for Conservation of Nature (IUCN 2015) and extensive genetic studies reveal a high extinction risk of the two remaining populations (Casas-Marce et al. 2013, Palomares et al. 2012). To support and restore wild populations of the Iberian lynx, a captive breeding program has been initiated in centers of Spain and Portugal with ongoing breeding and reintroduction of animals (Simón et al. 2012). Unraveling the function and regulation of perCL in this species is a necessary prerequisite for successful development of ovulation induction protocols, which could enhance the performance of captive breeding programs and offer more opportunities for the survival of this beautiful animal (Pelican et al. 2006).

1.7 Focus of the study

Reproduction is undoubtedly a key to species survival and is driven by a combination of factors that may differ even within one family. Understanding the basic mechanisms of feline reproduction and particularly species-specific differences in it is vital for the conservation and management of endangered felids. Moreover, the known high variations in patterns of mammalian reproduction exclude the possibility of full knowledge transfer from one species to another and demand basic studies in each genus.

This study is a part of a bigger project of our group, which aims to investigate the involvement of known luteotropic and luteolytic factors in the formation, maintenance and regression of the

feline CL, as well as their potential contribution to the CL physiological persistence. Current work presents basic histological and endocrinological studies on the domestic cat and two lynx species, Iberian and Eurasian lynx. To further investigate potential molecular pathways of the CL function and regulation in these species, this work centers around the systems of apoptosis and steroid receptivity in the CL. The focus of the presented three chapters can be shortly described as follows:

Chapter I, "The *corpus luteum* of the domestic cat: Histologic classification and intraluteal hormone profile" – here the general histomorphological and endocrine events are described throughout the domestic cat luteal phase. This study was initiated due to a dramatic lack of knowledge on the changes in histology of domestic cat CL, and incomplete information on the patterns of intraluteal P4 and estrogens. Moreover, the results of this study were necessary for any further research on potential luteotropic and luteolytic factors in the feline CL, because they provided the histomorphological staging of the domestic cat luteal phase, correlation with intraluteal steroids and a basis for comparative staging of lynx CL.

Chapter II, "Apoptosis-related factors in the luteal phase of the domestic cat and their involvement in the persistence of *corpora lutea* in lynx" – this study introduces the histomorphological staging and intraluteal steroid content of the obtained lynx CL, which allows further determination of the structural and functional state of persistent CL before and after ovulation. Selected apoptosis-related factors that are involved in the luteal phase of other species are analyzed for the first time in CL of the domestic cat and lynx. Investigation of these factors contributes to the search of main regulators of the feline luteal phase and allows initial analysis of their possible role in persistent CL of lynx.

Chapter III, "Progesterone, estrogen and androgen receptors in the *corpus luteum* of the domestic cat (*Felis catus*), Iberian lynx (*Lynx pardinus*) and Eurasian lynx (*Lynx lynx*)" – the same CL samples from domestic cats and lynx are analyzed for their receptivity to sex steroids.

INTRODUCTION

This study further discusses regulation of the feline luteal phase and potential mechanisms of the CL persistence in lynx, this time from the perspective of steroid action in the CL.

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2 CHAPTER I

The *corpus luteum* of the domestic cat: Histologic classification and intraluteal hormone profile

Chapter is presented in a form of reference.

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Authors: Amelkina O, Braun BC, Dehnhard M, Jewgenow K.

Contribution of each co-author to the manuscript:

OA performed sample collection, carried out histology and histobiochemistry, performed histomorphological characterization, participated in EIA (Enzyme Linked Immunosorbent Assay), conducted statistical analysis, interpreted the data and wrote the article.

BCB participated in study design and discussion of the results

MD provided hormonal analysis (EIA)

KJ supervised the study and contributed to its design, and discussion of the results

All co-authors revised the paper.

3 CHAPTER II

Apoptosis-related factors in the luteal phase of the domestic cat and their involvement in the persistence of *corpora lutea* in lynx

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Authors: Amelkina O, Zschockelt L, Painer J, Serra R, Villaespesa F, Braun BC, Jewgenow K.

Contribution of each co-author to the manuscript:

OA carried out CL collection (cat) and histological analysis (cat and lynx), staged the samples based on their histomorphological appearance, performed molecular studies, i.e., RNA isolation, RNA quality control, RT-qPCR establishment and measurements, analyzed obtained sequences and designed primers, carried out immunohistochemistry, participated in EIA for lynx CL, conducted statistical analysis, interpreted the data and wrote the article.

LZ contributed to molecular studies, i.e. RNA isolation and RT-qPCR measurements

JP collected ovarian samples of Eurasian lynx, Norway

RS collected ovarian samples of the Iberian lynx, Portugal

FV collected ovarian samples of the Iberian lynx, Spain

BCB supported sequence analyses, participated in a study design and discussion of the results

KJ supervised the study and its design, and participated in a discussion of the results

All co-authors revised the paper.

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RESEARCH ARTICLE

Apoptosis-Related Factors in the Luteal Phase of the Domestic Cat and Their Involvement in the Persistence of *Corpora Lutea* in Lynx

Olga Amelkina[1]*, Lina Zschockelt[1], Johanna Painer[1], Rodrigo Serra[2], Francisco Villaespesa[3], Beate C. Braun[1], Katarina Jewgenow[1]

1 Department of Reproduction Biology, Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany, **2** Iberian lynx captive breeding program, Centro Nacional de Reprodução de Lince Ibérico, Silves, Portugal, **3** Iberian lynx captive breeding program, Centro de Cría de Lince Ibérico El Acebuche, Parque Nacional de Doñana, Huelva, Spain

* olga.amelkina@gmail.com

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Abstract

The *corpus luteum* (CL) is a transient gland formed in the ovary after ovulation and is the major source of progesterone. In the Iberian and Eurasian lynx, CL physiologically persist after parturition and retain their capacity to produce progesterone, thus suppressing the ovarian activity. This unique reproductive characteristic has a big impact on the success of assisted reproduction techniques in the endangered Iberian lynx. The mechanisms behind CL persistence are not yet understood and require extensive studies on potential luteotropic and luteolytic factors in felids. Because the apoptosis system has been shown to be involved in structural regression of CL in many species, we aimed to investigate the capacity of perCL to undergo apoptosis. In addition, we performed initial studies on the apoptosis system in the luteal phase of the domestic cat. No previous research on this system has been made in this species. Our factors of interest included agents of the intrinsic apoptosis pathway, *i.e.*, pro-survival B-cell CLL/lymphoma 2 (BCL2) and pro-apoptotic BCL2-associated X protein (BAX), the executioner caspase-3 (CASP3), as well as of the extrinsic pathway, *i.e.*, pro-apoptotic receptor FAS, and tumor necrosis factor (TNF) and its receptors (pro-apoptotic TNFRSF1A and pro-survival TNFRSF1B). We analyzed the relative mRNA levels of these factors, as well as protein localization of CASP3 and TNF during stages of pregnancy and the non-pregnant luteal phase in CL of domestic cats. The same factors were investigated in freshly ovulated CL (frCL) and perCL of Iberian and Eurasian lynx, which were histologically analyzed. All factors were present in the CL tissue of both domestic cat and lynx throughout all analyzed stages. The presence of pro-apoptotic factors *BAX*, *CASP3*, *FAS* and *TNFRSF1A* in perCL of the Eurasian and Iberian lynx might indicate the potential sensitivity of perCL to apoptotic signals. The expression of pro-survival factors *BCL2* and *TNFRSF1B* was significantly higher in perCL compared to frCL of studied Iberian lynx, suggesting the potential involvement of these factors in the structural integrity of perCL. In both Iberian lynx and pregnant and non-pregnant domestic cats, the expression of *TNFRSF1A* was significantly higher in forming CL compared to other stages, suggesting the conserved involvement of this factor in the tissue reorganization during formation of the

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feline CL. The mRNA levels of *CASP3* and *TNFRSF1B* were highest during regression stages of domestic cat CL. The current study provides initial results on the possible involvement of the apoptosis system in the structure and function of the feline CL and in its physiological persistence.

Introduction

The *Lynx* genus includes four species that inhabit different parts of the world: the Eurasian lynx (*Lynx lynx*) in the broad range of Europe and Asia, the Canada lynx (*Lynx canadensis*) and the bobcat (*Lynx rufus*) in North America, and the Iberian lynx (*Lynx pardinus*) in the Iberian Peninsula. Eurasian, Canada and Iberian lynx are monoestrous, *i.e.*, mate only once in a year, with breeding seasons in January–April, February–April and January–February, respectively [1–4]. The bobcat is seasonally polyestrous and may ovulate up to three times during the breeding season [5].

All three monoestrous lynx species exhibit a unique reproductive characteristic associated with the *corpus luteum* (CL), a transient gland that forms in the ovary after ovulation and supports pregnancy *via* its production of progesterone [6]. It has been widely shown among different species that, in the event of a non-pregnant cycle or at the end of pregnancy, the CL regresses from the ovarian tissue and a new cycle is initiated [7, 8]. However, the situation is markedly different for the lynx. Studies on the lynx ovarian cycle revealed the presence of CL outside the reproductive season in Iberian and Eurasian lynx [9]. More recent findings indicate that CL of the Eurasian lynx morphologically persist in the ovary for at least two years [10]. Furthermore, such persistent CL (perCL) continue to produce progesterone and thus exhibit functional persistence, leading to a stage called prolonged diestrus between cycles [10, 11]. Studies on the Canada lynx also suggest a similar luteal pattern, based on constant fecal progesterone concentrations [1]. The bobcat seems to exhibit morphological persistence of CL, however, their functional condition still needs to be investigated [12, 13]. To summarize, CL persist morphologically and functionally after parturition and weaning in all three monoestrous lynx species: Iberian, Eurasian and Canada lynx.

There is as yet no certainty about the role of perCL in lynx reproduction, nor are there any reports so far on the possible molecular mechanisms involved. It has been hypothesized that perCL may be advantageous in lynx species by supporting subsequent pregnancies as additional sources of progesterone [14] and/or by securing a monoestrous cycle *via* progesterone suppression of ovarian activity [15]. Such suppression of any late ovulations would ensure the birth and weaning of cubs during the most favorable time of the year [10]. It is unclear how the monoestrous lynx enters a new ovarian cycle every year despite the constant presence of active perCL and, therefore, it is not possible to design any protocols of ovulation induction for these species. An attempt to overcome the CL persistence by administrating a commonly luteolytic prostaglandin $F_{2\alpha}$ led to a temporary decrease in progesterone concentration, but structurally perCL remained in the ovary [16]. The lynx unique reproductive characteristic, therefore, makes standard protocols for assisted reproduction inapplicable in monoestrous lynx and may critically lower the success of breeding programs for these species. This greatly affects the Iberian lynx, which until recently had been listed as critically endangered by the International Union for Conservation of Nature [17] and for which extensive genetic studies revealed a high extinction risk of the two remaining populations [18, 19]. To support and restore wild populations of the Iberian lynx, a captive breeding program has been initiated in the centers of Spain

and Portugal with ongoing breeding and reintroduction of animals [20]. Unraveling the function and regulation of perCL, and particularly the mechanisms of its regression, could enhance the performance of captive breeding programs and offer more opportunities for the Iberian lynx survival.

The research on the lynx or any other wild felid is complicated by the limited access to these species. The sampling of reproductive tissue is rare and depends heavily on a chance, be it *post-mortem* sampling after legal hunting or natural/accidental death of an animal, or an ovariohysterectomy of captive lynx for medical and management reasons. Because of this, the more accessible felid, domestic cat (*Felis catus*), becomes an irreplaceable model species. The domestic cat is seasonally polyestrous, and after ovulation queens can either enter a period of pregnancy (approximately 65 days [21]) or a non-pregnant luteal phase (approximately 40 days [22]). In both scenarios, elevated serum and intraluteal progesterone concentrations decrease towards the end of the luteal phase, allowing initiation of a subsequent ovarian cycle [23, 24]. Molecular studies on the domestic cat CL have been limited to the investigation of enzymes of the luteal steroidogenesis system [25]. To contribute to the knowledge on the mechanisms behind formation, maintenance and regression of the feline CL, we initiated a detailed molecular study on potential luteotropic and luteolytic factors in the domestic cat [26] and lynx [27]. In the present work, we focus on the mechanisms of structural regression of the CL in the domestic cat and in two species of lynx, Iberian and Eurasian.

Luteal regression (luteolysis) is commonly required for the initiation of a new follicular cycle. The literature distinguishes between two aspects of luteolysis, functional and structural, with the first condition usually preceding the latter one [8]. Both aspects of luteolysis are governed by species-specific luteal factors, however, structural luteal regression *via* apoptosis-related factors is common to most of the species studied to date [7, 28–30]. Apoptosis, or programmed cell death, can be divided into intrinsic and extrinsic pathways. The intrinsic signaling cascade is generally activated by apoptotic stimuli within a cell. Members of the B-cell CLL/lymphoma 2 (Bcl-2) family transduce the signal within the cell by alterations in the mitochondrial outer membrane (MOM): *e.g.*, the pro-apoptotic factor BCL2-associated X protein (BAX) resides in the cytosol or on MOM itself and, once activated by the BH3-only proteins and oligomerized, creates permeability of the MOM [31, 32]. This permeability leads to release of cytochrome c from the inner mitochondrial membrane and thus causes activation of caspase-3 (CASP3) which, through cleavage of essential proteins, executes the final step of cell death [33]. The pro-survival factor BCL2 can inhibit the death signal *via* translocation of BAX from MOM, or by sequestering BH3-only proteins and preventing them from activating BAX, or by binding to activated BAX and preventing its oligomerization [31]. All the mentioned factors were shown to be involved in luteolysis in rodents, cattle and primates [34–39].

The extrinsic signaling cascade is activated by extracellular signals *via* cytokines, which include members of the tumor necrosis factor (TNF) super family, *e.g.*, TNF (also known as TNF superfamily, member 2 and TNF-alpha) and Fas ligand, and their cell surface receptors, *e.g.*, TNF receptor superfamily, member 1A (TNFRSF1A; previously known as TNFR1), TNFRSF1B (previously known as TNFR2) and Fas cell surface death receptor (FAS; also known as CD95) [40]. The FAS/Fas ligand system is known to transduce the death signal *via* protease cascade and was shown to be required for luteolysis in many species [41–44]. Another cytokine, TNF, exhibits pleiotropic signals in the CL tissue and its action might be dependent on its concentration, the local environment, the stage of the cycle and the type of receptor it binds to [43, 45–47]. While TNFRSF1A contains the death domain and can transduce the death signal, in contrast, TNFRSF1B does not possess the death domain and can act as a pro-survival factor [40, 48, 49]. Presented list of factors was found to play an important role throughout the luteal phase of many species, including representatives of rodents, cattle and

primates [42, 48–53]. Both intrinsic and extrinsic pathways lead to activation of the caspase family, *i.e.*, caspase-9 and -8 respectively, and subsequently the final executioners caspase-3, -6 and -7 [54]. Short description of each factor can be found in Table 1.

We had three hypotheses that might explain the prolonged structural integrity of perCL in lynx: (i) either CL persist due to their limited capacity to undergo apoptosis; or (ii) there is no luteolytic signal and, therefore, no activation of the apoptotic cascade; or (iii) the transducing luteolytic signal is blocked on the downstream of the apoptotic cascade. To study these potential mechanisms, we aimed to first investigate the capacity of perCL to undergo apoptosis. As a necessary basis, we studied the involvement of selected apoptosis-related factors, *i.e.*, *BCL2*, *BAX*, *CASP3*, *FAS*, *TNFRSF1A*, *TNFRSF1B* and *TNF*, in the luteal phase of the domestic cat. Then, we analyzed the presence of these factors in perCL of Iberian and Eurasian lynx and compared the capacity of freshly ovulated (fr) CL and perCL to express them in the Iberian lynx.

Materials and Methods

All chemicals in the study were purchased from Sigma-Aldrich (Taufkirchen, Germany), unless stated otherwise and were of the highest purity available.

2.1 Ethics statement

The methods applied, and the study-design, were approved by the Internal Committee for Ethics and Animal Welfare of the Leibniz Institute for Zoo and Wildlife Research in Berlin, Germany (Permit numbers: 2010-10-01 and 2011-01-01). The Norwegian Experimental Animal Ethics Committee approved the collection of the ovarian tissue from hunted animals (Permit number: 2010/161554). The tissue from late pregnancy castration of domestic cats were obtained from the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn, Poland, and all procedures were approved by the Local Animal Care and Use Committee in Olsztyn, Poland (No. 41/2007/N and 61/2010/DTN).

2.2 Animals and tissue collection

Ovaries of domestic cats were obtained from local animal shelters and clinics after ovariohysterectomy for the purpose of permanent contraception. The reasons to perform ovariohysterectomy were not related to the study. Samples were transported in MEM-HEPES medium, supplemented with 3 mg/mL BSA (Merck Millipore, Darmstadt, Germany) and 1x Antibiotic Antimycotic Solution. Transportation was at 4°C, and ovaries were processed immediately after arrival at the laboratory (2–4 hours after surgery). The isolation process and consequent staging of CL is described in Amelkina *et al.* [24]. In brief, CL from each cat were either fixed in Bouin's solution for histologic analysis or plunged into liquid nitrogen for RNA isolation. In the case of pregnancy, the day was assessed by the diameter of the gestation chamber [55], the crown-rump length of a foetus [56] or by the stage of a pre-implantation embryo [57]. The pre-implantation period (n = 6) included samples from days 2 to 6 and day 10 *post-coitum*; the post-implantation period (n = 11) included samples from days 14 to 36 *post-coitum*; finally, the CL regression stage (n = 5) was represented by samples from days 38, 39, 48 and week 9 *post-coitum*. The absence of embryos in the oviducts or uteri indicated a non-pregnant luteal phase. In such cases, based on their histologic appearance, each CL was classified as the stage of: formation (n = 9), development/maintenance (n = 13), early regression (n = 14), late regression (10) or *corpus albicans* (CA; n = 4). The histologic classification is described in detail in Amelkina *et al* [24] and includes parameters of: cell shape, type and degree of vacuolation, nucleus

Table 1. List of analyzed factors, sequences of PCR primers used for sequence analysis and expression studies, annealing temperatures, and product sizes. bp, base pair; fw, forward; rv, reverse; T_A, annealing temperature; a, used for sequence analysis; b, used for gene expression studies. Intrinsic/extrinsic pathway refers to the apoptosis pathways

Factor	Short description	GenBank ID	Species	Primer sequence 5'– 3'	T_A (°C)	Product size (bp)	Use
BCL2	Bcl-2 family, intrinsic pathway	DQ926871.1 KP826765*	*Felis catusLynx pardinus*	*BCL2* Fw: GAG ATG TCC AGC CAG CTG*BCL2* Rv: TAG GCA CCC AGG GTG ATC	53	365	a
	pro-survival function			*BCL2* qFw: GGA GGA TTG TGG CCT TCT*BCL2* qRv: GGT TCA GGT ACT CAG TCA TCC AC	54.5	143	b
BAX	Bcl-2 family, intrinsic pathway	DQ926869.1KP862666*	*Felis catusLynx pardinus*	*BAX* Fw: CAG CTC TGA GCA GAT CAT G*BAX* Rv: TGG TGG CCT CAG CCC ATC T	53	595	a
	pro-apoptotic function			*BAX* qFw: CCG ATG GCA ACT TCA ACT GGG*BAX* qRv: GAT GGT CAC TGT CTG CCA CGT C	63	244	b
CASP3	protease, intrinsic pathway	NM_001009338.1KP294340*	*Felis catusLynx pardinus*	*CASP3* Fw: GTG TGC GTT AGA AGT ACC*CASP3* Rv: GTT CTT TTG TGA GCA TAG ACA	53	836	a
	executioner function			*CASP3* qFw: ACC GGC AAA CCC AAA CTC*CASP3* qRv: CTG ACA GGC GAT GTC ATC C	60.5	91	b
FAS	cytokine receptor, extrinsic pathway	NM_001009314.1KP670850*	*Felis catusLynx pardinus*	*FAS* Fw: CCG TTG GCT GAT ACT TAC C*FAS* Rv: CGT GTT TGC AGT TTC CAT TC	53	952	a
	generally pro-apoptotic function			*FAS* qFw: GAA CGC TAC CGA AGG GGA*FAS* qRv: GTC GGC AGC TTT TCG TGT	59.5	100	b
TNFRSF1A	cytokine receptor, extrinsic pathway	NM_001009361.1KP670852*	*Felis catusLynx pardinus*	*TNFRSF1A* Fw: GCA GGA AGA ACC AGT ACC GG*TNFRSF1A* Rv: CCG TTC TGC AGC TCC AGC C	53	846	a
	pro-apoptotic function			*TNFRSF1A* qFw: AGA GTG TAC GAA GTT GTG CG*TNFRSF1A* qRv: AGC TTG GAC TTC CGC CGT T	60.5	159	b
TNFRSF1B	cytokine receptor, extrinsic pathway	KP670853*KP670854*	*Felis catusLynx pardinus*	*TNFRSF1B* Fw: CCC CCG GGC TTC GGC GT*TNFRSF1B* Rv: CTT GGA CAA GGG GAC CTG CT	53	799	a
	pro-survival function			*TNFRSF1B* qFw: AGC AGC TCC CTG GAG AGC *TTNFRSF1B* qRv: GTG ACA TTG ACC TGG GTC C	60.5	168	b
TNF	cytokine, extrinsic pathway	NM_001009835.1KP670851*	*Felis catusLynx pardinus*	*TNF* Fw: CAT GAG CAC TGA AAG CAT GA*TNF* Rv: TCA CAG GGC AAT GAT CCC A	53	703	a
	pleiotropic effects			*TNF* qFw: AGA GCT CCC ACA TGG CCT*TNF* qRv: GGC TCA GCC ACT GGA GTT	59.5	137	b

* GenBank sequences were obtained in this study

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condition, and the ratio of non-steroidogenic to luteal cells. Listed n-values represent the number of animals per analyzed stage; each animal is represented by one CL.

Ovaries of Iberian lynx were collected in the scope of the Iberian lynx captive breeding program at the Centro de Cría de Lince Ibérico El Acebuche, Parque Nacional de Doñana, Huelva, Spain and Centro Nacional de Reprodução do Lince Ibérico, Silves, Portugal. Two animals were ovariohysterectomized for permanent contraception seven days after ovulation was

induced by natural mating in February, 2013. Ovariohysterectomy was initiated due to the medical conditions of animals (repeated caesarean sections and mammary tumor risk) and was not related to the study. In one animal (Iberian lynx 1, nine years old), embryos were flushed from the uterus, indicating the pre-implantation stage of pregnancy. Unfertilized oocytes were flushed from the second animal (Iberian lynx 2, eleven years old), thus indicating a non-pregnant luteal phase. The CL were isolated immediately after surgery and their morphological appearance was noted. The presence of ovulation scars allowed distinguishing CL of fresh ovulation (frCL) from CL of previous cycle/s (perCL), supplemented later by histologic analysis of both types (see Results). Each CL was dissected and pieces were fixed in Bouin's solution (for histologic analysis) or placed in RNA-later solution (RNA isolation; Qiagen GmbH, Hilden, Germany) or liquid nitrogen (hormone analysis).

Ovaries of free-ranging Eurasian lynx were collected freshly *post-mortem* from animals hunted legally during the national hunting quota for management purposes in Norway (n = 5 animals). The period of collection was the beginning of the breeding season prior to mating (February, 2011; the breeding season for Eurasian lynx in Norway is February to early April [2]). After dissection, samples were immediately fixed in Bouin's solution (for histologic analysis) or placed in RNA-later (RNA isolation) or Allprotect Tissue Reagent (hormone analysis; Qiagen GmbH, Hilden, Germany) solutions. Based on the pre-breeding period of collection and the absence of frCL, embryos and placental scars of a recent pregnancy, all isolated CL were classified as perCL from previous cycle/s.

To demonstrate the steroidogenic activity of the isolated CL, intraluteal concentrations of progesterone and estrogens were determined for Iberian and Eurasian lynx by enzyme immunoassay per wet weight of CL, as described and validated previously [11, 24].

2.3 RNA isolation and cDNA synthesis

Up to 26 mg of CL tissue was homogenized in homogenization tubes (100 μl RNA lysis buffer, 1.4/2.8 mm ceramic beads) at 5000 rpm for 2 x 25 sec (Precellys 24 homogenizer, Bertin Technologies, Montigny-le-Bretonneux, France). Total RNA was extracted using the innuSPEED Tissue RNA/innuPREP DNase I Digest Kit (Analytik Jena AG, Jena, Germany). The NanoDrop ND-1000 (PEQLAB Biotechnologie GmbH, Erlangen, Germany) was used to assess the concentration and purity of isolated RNA. Additional control of RNA quality and integrity was performed *via* microfluidic analysis using the Bioanalyzer (Agilent Technologies Deutschland GmbH, Boeblingen, Germany); RNA integrity number (RIN) values were above 7.0 for cat samples and above 6.1 for Iberian and Eurasian lynx samples. From 1 to 2.5 μg of isolated RNA was reverse transcribed into single-stranded (ss) cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham MA, USA). No reverse transcriptase was added to the negative control to verify the absence of genomic DNA contamination.

2.4 Sequencing

Primers for the polymerase chain reaction (PCR) were purchased from BioTeZ Berlin Buch GmbH (Berlin, Germany) and were designed based on *Felis catus* gene sequences listed in NCBI database (see accession numbers in Table 1). The NCBI-sequence for *TNFRSF1B* was only predicted by automated computational analysis (XM_003989583.2) and thus it was analyzed in this study too. Primer information is listed in Table 1. Based on feline ss cDNA templates of luteal, ovarian or placental origin, partial cat and lynx cDNA sequences were amplified using the Expand High FidelityPLUS PCR system (Roche Diagnostics Deutschland GmbH, Mannheim, Germany), as described by Braun *et al.* [58]. For both cat and lynx, the PCR conditions were: 94°C for 2 min; followed by 35 cycles of denaturation at 94°C for 45 sec,

annealing at 53°C for 45 sec, elongation at 72°C for 80 sec; and final elongation at 72°C for 7 min. Purified PCR products were ligated to the pJET 1.2 vector (Thermo Fisher Scientific) and transfected in JM109 cells (Promega GmbH, Mannheim, Germany) for cat *CASP3*, *FAS* and *TNF* or ligated to the pCR4-TOPO TA vector and transfected in TOP10 cells (both Life Technologies GmbH, Darmstadt, Germany) for lynx *CASP3*, *FAS*, *TNF* and cat/lynx *TNFRSF1A* and *TNFRSF1B*. Positive clones or purified PCR products (*BCL2* and *BAX*) were sequenced by the Services in Molecular Biology GmbH (Dr M. Meixner, Brandenburg, Germany).

2.5 Quantitative real-time PCR

Primers for quantitative real-time PCR (qPCR) were based on cat and lynx sequences identified in this study (Table 1). The qPCR was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories GmbH, Munich, Germany), as published by Braun *et al.* [59]. In brief, diluted ss cDNA (4 µl, corresponding to 2 or 10 ng of total RNA for genes of interest, or 4 ng for reference genes) were analyzed in a 10 µl reaction volume including SsoFast EvaGreen Supermix (Bio-Rad Laboratories GmbH). The qPCR conditions were: 98°C for 2 min and 40 cycles of 8 sec at 98°C and 8 sec at different annealing temperatures (Table 1). Quantification of qPCR products was performed using the CFX Manager Software 1.6 (Bio-Rad Laboratories GmbH). Serial dilutions of plasmid DNA were used for calibration. Glutaminase (*GLS*; for domestic cat, JQ424891), TATA box binding protein (*TBP*; for domestic cat, JQ424890; for lynx, JX993351), β-actin (*BACT*; for domestic cat, AB051104; for lynx, KM458620), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH;* for lynx, KM458621) and ribosomal protein S7 (*RPS7*; for lynx, JX993349) were validated as optimal reference genes in feline CL with the qbasePLUS software (Biogazelle, Zwijnaarde, Belgium; [60]) and were used for normalization. A multiple normalization factor was calculated for individual CL referring to Vandesompele *et al.* [61].

2.6 Histologic analysis of CL and protein localization

Tissues fixed in Bouin's solution were dehydrated, embedded in paraffin following standard procedures and sectioned at 3 µm. Subsequent histologic evaluation was performed under a light microscope fitted with a digital camera (Jenoptik ProgRes C3, Jena, Germany).

To determine the histomorphological state of CL, samples from Iberian and Eurasian lynx were routinely stained with haematoxylin and eosin. The histomorphological analysis of cat samples was performed in our previous study [24].

The localization of CASP3 protein in luteal tissue was assessed by an affinity-purified rabbit anti-human/mouse CASP3 reactive antibody (Cat. AF 835; R&D Systems, Wiesbaden, Germany), used in a previous study on the domestic cat [62]. According to the manufacturer, this antibody recognizes an active form of CASP3. Localization of TNF protein was assessed by a goat polyclonal anti-human TNF antibody (Cat. No. sc-1350; Santa Cruz Biotechnology, Dallas TX, USA). Immunohistochemistry was performed as described in Braun *et al* [58]. In brief, sectioned CL tissue mounted on microscope slides (Superfrost Plus, Thermo Scientific, Braunschweig, Germany) was deparaffinized in Roti-Histol (Carl Roth GmbH, Karlsruhe, Germany) and rehydrated in decreasing concentrations of ethanol. Slides were subsequently incubated in boiling citrate buffer (11 mM, pH 6.0) for 15 min and in 3% H_2O_2/methanol solution for 10 min. Then, sections were blocked with 5% BSA in PBS for 1 h at 37°C. Antibody against CASP3 was diluted in PBS 1:500; antibody against TNF was diluted in 1% BSA in PBS 1:200. Sections were subsequently washed with PBS-Tween 0.1%, incubated with either peroxidase-conjugated anti-rabbit or anti-mouse EnVisionC reagent (ready to use solution; Dako Agilent Technologies, Glostrup, Denmark) for 1 h at 23°C and colour-detected with diaminobenzidine

(DAB) substrate chromogen solution (Dako Agilent Technologies). Finally, sections were counterstained with hematoxylin, dehydrated in increasing concentrations of ethanol, and covered with mounting medium and coverslips.

2.7 Statistical analysis

Statistical analysis was performed with the R software package (R: A language and environment for statistical computing, version 3.0.0, Vienna, Austria). For the domestic cat, the Kruskal-Wallis rank sum test was used to determine changes in relative mRNA levels throughout pregnant and non-pregnant luteal phases. The Wilcoxon rank sum test was used for *post-hoc* pairwise comparison of stages (P-value adjustment: Benjamini-Hochberg). Each animal was represented by one CL. Numbers of animals per stage can be found above in 2.2. The stage of CA was not included in the statistical analysis, because its origin (pregnant or non-pregnant luteal phase) in the ovary was unknown. For Iberian lynx, the Mann-Whitney U-test was used to determine changes in relative mRNA levels between frCL and perCL in each animal. Sample size for Iberian lynx was the following: Iberian lynx 1, n = 3 for frCL and n = 8 for perCL; Iberian lynx 2, n = 5 for frCL and n = 6 for perCL. Probability (P) values less than 0.05 were considered statistically significant. SigmaPlot 10.0 (Systat Software Inc., San Jose CA, USA) was used to visualize the statistical results *via* boxplots.

Results

In this study, we obtained partial feline gene sequences for the analyzed factors (Table 1).

3.1. Domestic cat

3.1.1 Pregnancy. Throughout the pregnant luteal phase, no significant changes were observed in mRNA levels of *BCL2*, *BAX*, *CASP3* and *TNF* (Fig 1A–1C/1G). Relative *FAS* mRNA levels were higher during CL regression compared to the post-implantation period (P = 0.026; Fig 1D). Gene expression of TNF receptors changed significantly during pregnancy: *TNFRSF1A* (Fig 1E) relative mRNA levels were higher during the pre-implantation period compared to post-implantation (P = 0.0019) and CL regression (P = 0.013); *TNFRSF1B* (Fig 1F) relative mRNA levels were higher during CL regression compared to post-implantation (P = 0.055).

3.1.2 Non-pregnant luteal phase. Throughout the non-pregnant luteal phase, relative mRNA levels of *BCL2*, *CASP3*, *TNFRSF1A* and *TNFRSF1B* changed significantly, while no significant changes were observed for *BAX*, *FAS* and *TNF* (Fig 1). For *BCL2* (Fig 1A), relative levels of mRNA changed throughout the luteal phase (P = 0.017), but no significance was found in the *post-hoc* comparison. For *CASP3* (Fig 1C), relative mRNA levels during CL formation were lower compared to early regression (P = 0.0014) and late regression (P = 0.0038). Relative mRNA levels of *TNFRSF1A* (Fig 1E) were higher during CL formation compared to development/maintenance (P = 0.00023), early regression (P = 0.0013) and late regression (P = 0.00023). Finally, relative mRNA levels of *TNFRSF1B* (Fig 1F) during CL early regression were higher than during development/maintenance (P = 0.028).

The immunohistochemistry assay localized active CASP3 in luteal cells. The pattern differed individually for each animal and overall the expression of CASP3 protein could be found in at least one sample of every stage analyzed (Fig 2A–2D), except for CA (Fig 2E). Non-steroidogenic cells, presumably macrophages, were the only cells stained for TNF antibody in the CL (Fig 2F). The staining was rare and consistent with the results on mRNA level.

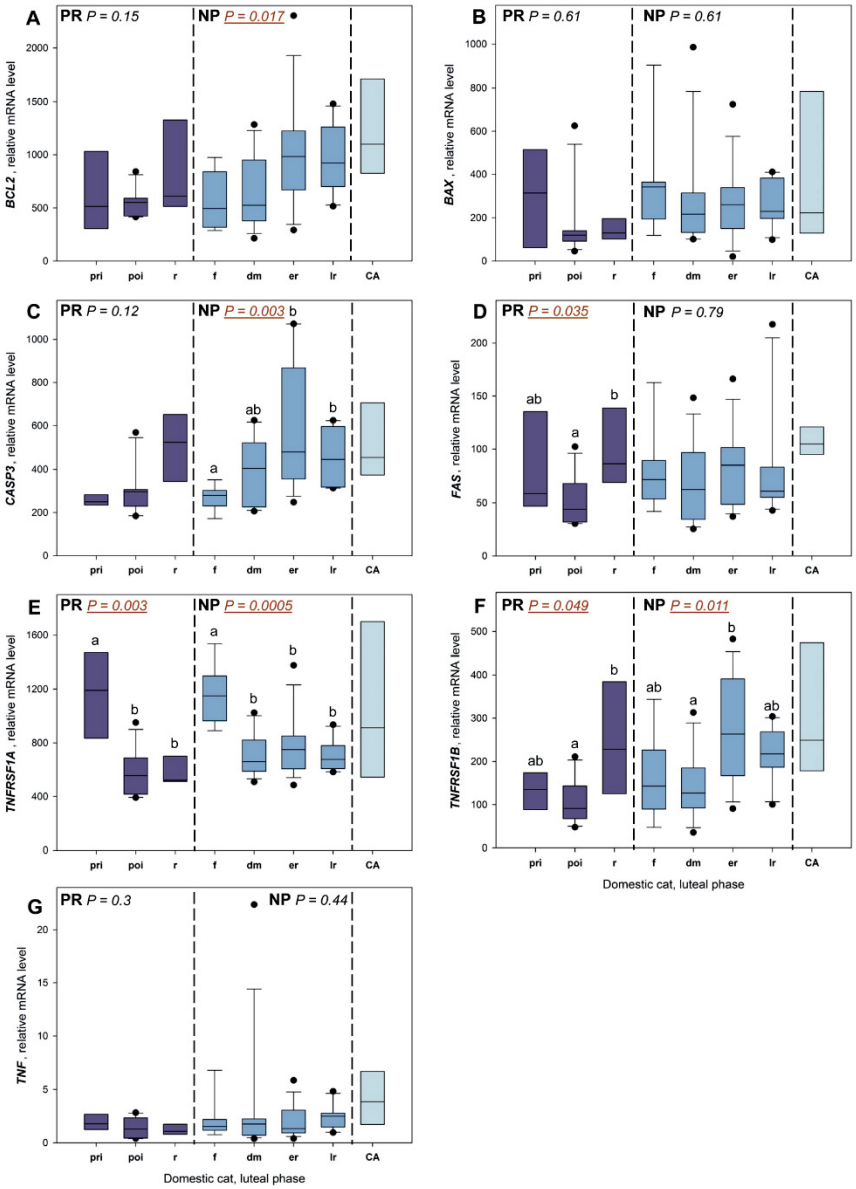

Fig 1. Intraluteal relative mRNA levels during pregnant and non-pregnant luteal phases in the domestic cat. Box plots depict the distribution of data; whiskers indicate maximum and minimum values; black dots indicate outliers; horizontal lines indicate the median; P-values are calculated from the Kruskal-Wallis rank sum test; letters (a, b) identify significant differences between stages and are calculated from *post-hoc* pairwise comparisons (P-value adjustment: Benjamini-Hochberg). PR: pregnancy; NP: non-pregnant luteal phase; pri: pre-implantation period; poi: post-implantation period; r: regression; f: formation; d/m: development/maintenance; er: early regression; lr: late regression; CA: *corpus albicans*.

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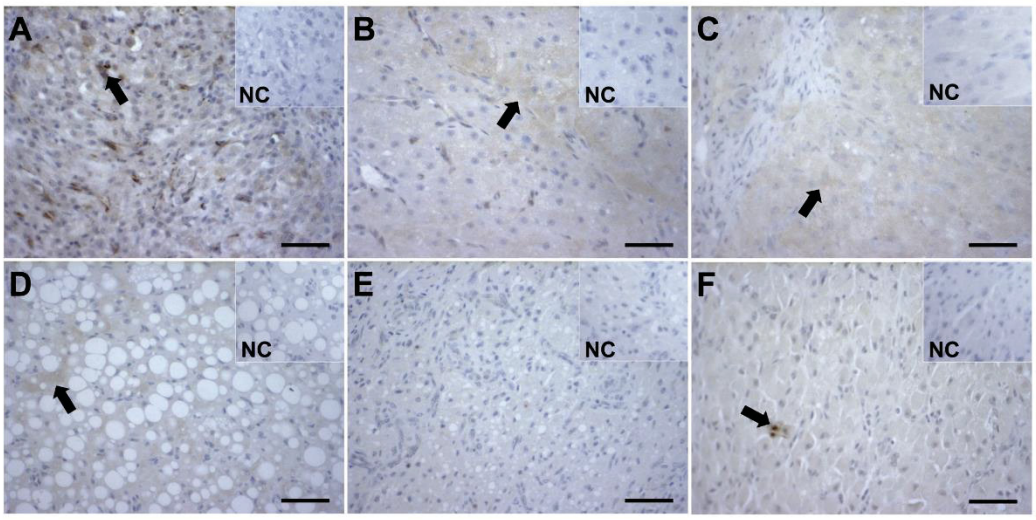

Fig 2. Immunohistochemical localization of CASP3 and TNF proteins in CL of the domestic cat. Active CASP3 was localized in luteal cells at the stages of: CL formation (A), development/maintenance (B), early regression (C) and late regression (D). The protein expression was consistently present throughout the CL life span, except for CA (E). TNF protein expression was identified in non-steroidogenic cells, presumably macrophages, but not in luteal cells (F). Arrows indicate positive staining. NC: negative control. Scale bar– 50 μm.

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3.2. Lynx

3.2.1 Iberian lynx, frCL *vs.* perCL post-mating. During dissection of ovaries, two types of CL could be clearly distinguished: frCL from a current ovulation and perCL from previous cycle/s (Fig 3). Fresh CL were paler and bigger than perCL and had an ovulation scar on their surface; perCL were smaller, darker and partly located inside of the ovary. Subsequent histologic analysis of these CL revealed a stage of formation for frCL and a stage of development/maintenance for perCL (Fig 4A and 4B). The formation stage of frCL exhibited processes of tissue reorganization, where predominantly small luteinizing cells varied in shape and size. The

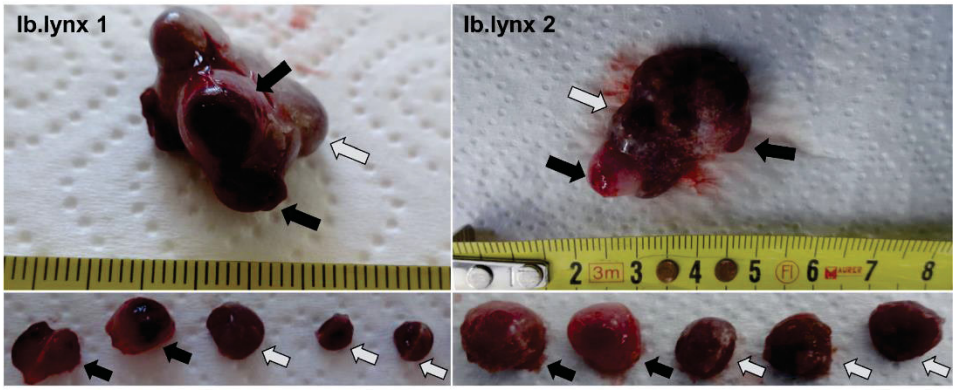

Fig 3. Morphological appearance of the Iberian lynx ovary. Ovaries of the Iberian lynx 1 and 2 (lb.lynx 1 and lb.lynx 2, respectively), containing frCL and perCL. Black arrows indicate frCL with ovulation scars, gray arrows indicate perCL from previous cycle/s. Scale–mm.

doi:10.1371/journal.pone.0143414.g003

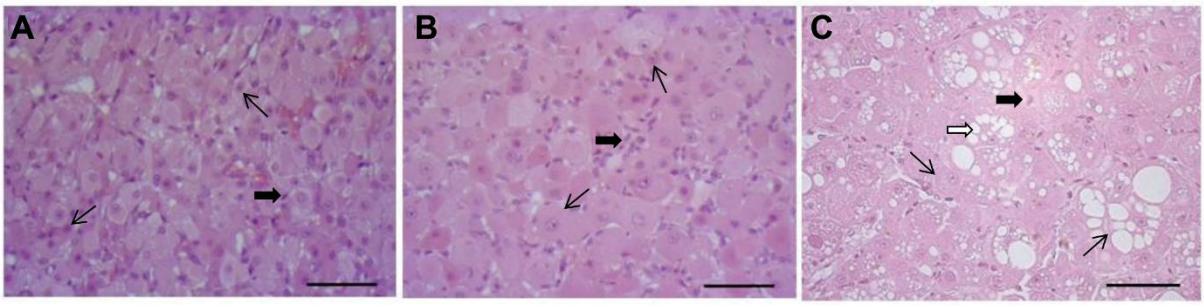

Fig 4. Haematoxylin and eosin stained sections of CL of Iberian and Eurasian lynx. A. Formation of the CL in Iberian lynx: frCL, seven days after natural mating. B. Maintenance of the CL in Iberian lynx: perCL of previous cycle/s after mating. C. Signs of regression in the CL of pre-mating Eurasian lynx: perCL of previous cycle/s during breeding season. Thin arrows indicate luteal cells. Thick black arrows indicate non-steroidogenic cells. White arrows indicate coarse vacuolation. Scale bar– 50 μm.

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development/maintenance stage of perCL showed no signs of tissue regression; luteal cells were large and polyhedral, exhibiting fine vacuolation. The histomorphological analysis was done with reference to the established histologic staging on the domestic cat [24]. Hormone analysis revealed very high concentrations of estrogens in frCL of Iberian lynx and maintained concentrations of progesterone in perCL (Table 2).

All the factors examined were expressed in frCL and perCL of Iberian lynx. Relative mRNA levels of *BCL2* (P = 0.012, Iberian lynx 1; P = 0.0043, Iberian lynx 2; Fig 5A), *FAS* (P = 0.048, Iberian lynx 1; P = 0.0086, Iberian lynx 2; Fig 5D), *TNFRSF1B* (P = 0.01, Iberian lynx 1; Fig 5D) and *TNF* (P = 0.02, Iberian lynx 1; Fig 5G) were all higher in perCL compared to frCL (Fig 5). Relative mRNA levels of *TNFRSF1A* were higher in frCL compared to perCL (P = 0.01, Iberian lynx 1; P = 0.004, Iberian lynx 2; Fig 5E). In both animals, no significant changes in relative mRNA levels of *BAX* and *CASP3* were observed (Fig 5B/5C).

Active CASP3 protein could not be localized in the analyzed CL tissue (example of negative staining in Fig 6A). Staining for TNF protein was observed in non-steroidogenic cells, presumably macrophages, in both types of CL, but also in some transforming cells of frCL (Fig 6B and 6C).

3.2.2 Eurasian lynx, perCL in breeding season before mating. Histomorphological analysis of perCL in Eurasian lynx revealed signs of luteal regression: coarse vacuolation could be observed across the luteal tissue (Fig 4D). This state was comparable to the histologic stage of early regression in domestic cat [24]. No CA stage of CL was observed. Concentrations of progesterone and estrogens were present in the luteal tissue (Table 2). All the factors examined were expressed in perCL of Eurasian lynx, including *BCL2* (relative mRNA level

Table 2. Intraluteal concentrations of estrogens and progesterone in Iberian and Eurasian lynx. Intraluteal concentrations of estrogens and progesterone in the domestic cat can be found in Amelkina *et al* [24]

Animal	Type of CL (n)	Estrogens, ng/gmean ± SD	Progesterone, μg/gmean ± SD
Iberian lynx 1	frCL (2)	6267.1, 5688.9	38.9, 31.8
	perCL (4)	241 ± 35	19.6 ± 5
Iberian lynx 2	frCL (3)	6517.6 ± 3795.3	20 ± 8.4 5
	perCL (4)	211.3 ± 201	16.3 ± 1.4
Eurasian lynx, n = 5	perCL (8)	10.1 ± 10.1	9.9 ± 5.8

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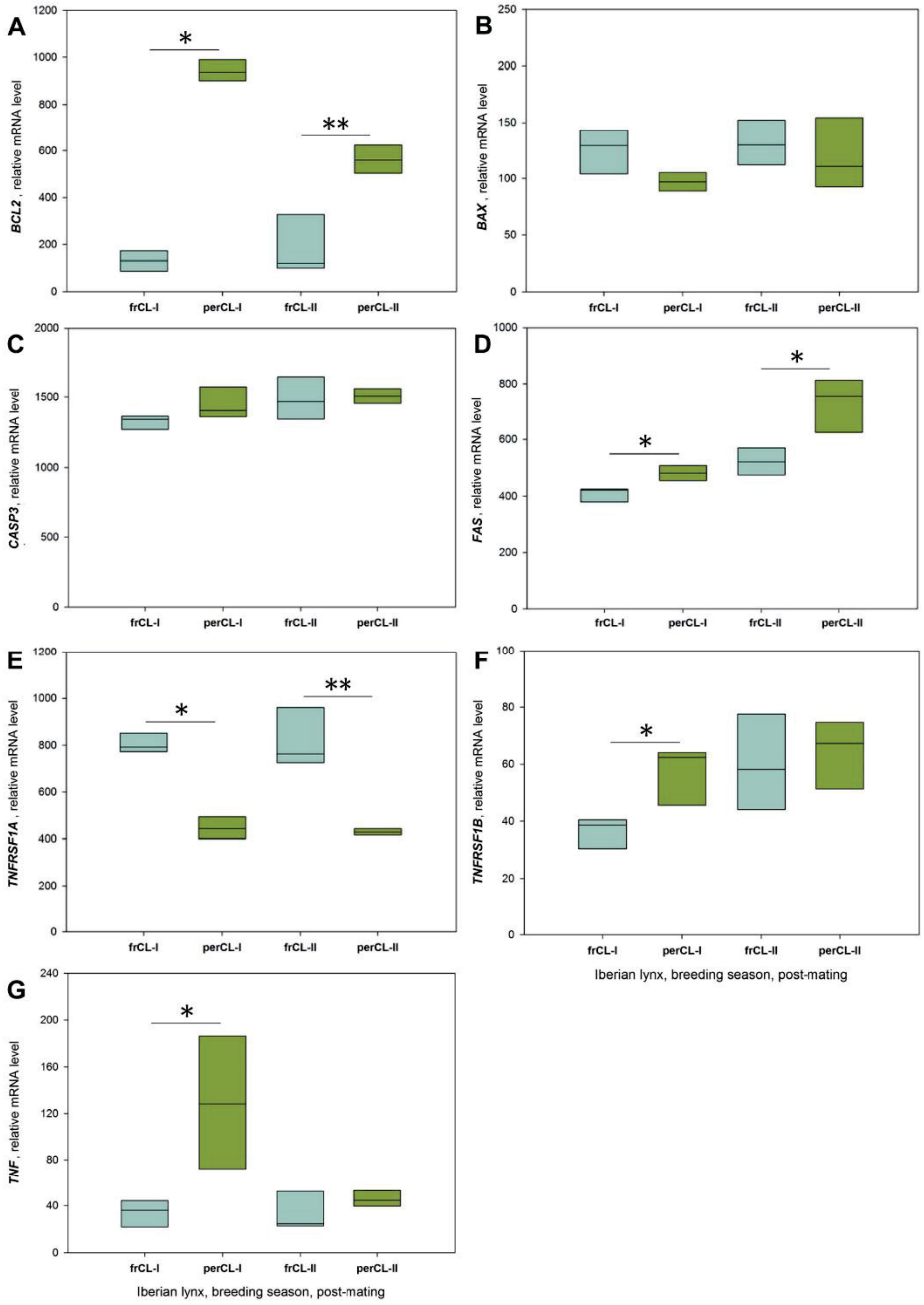

Fig 5. Intraluteal relative mRNA levels during the breeding season in post-mating Iberian lynx. Box plots depict the distribution of data; horizontal lines indicate the median. frCL-I/II: frCL from Iberian lynx1/2; perCL-I/II: perCL from Iberian lynx 1/2 * P < 0.05, ** P < 0.005; Mann-Whitney U-test.

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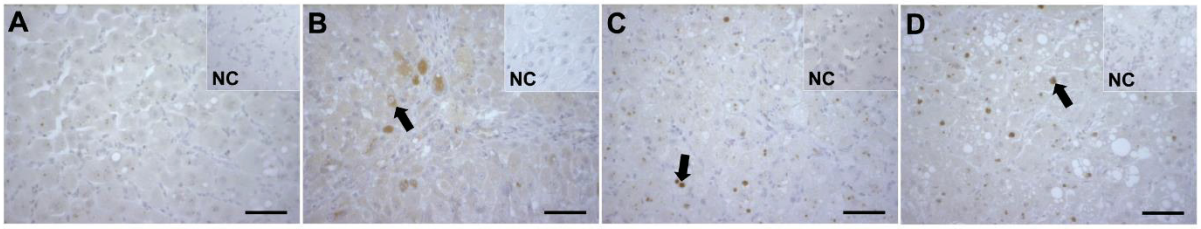

Fig 6. Immunohistochemical localization of CASP3 and TNF proteins in CL of Iberian and Eurasian lynx. A. Negative staining for active CASP3 in lynx CL tissue. B. frCL of Iberian lynx; strong staining for TNF protein in transforming cells (arrows). C. perCL of Iberian lynx; staining for TNF protein in non-steroidogenic cells, presumably macrophages (arrows). D. perCL of Eurasian lynx; staining for TNF protein in non-steroidogenic cells, presumably macrophages (arrows). Staining of droplets within the cells is unspecific. NC: negative control. Scale bar– 50 μm.

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726.4 ± 365.8), *BAX* (relative mRNA level 132.0 ± 46.9), *CASP3* (relative mRNA level 1092.3 ± 109.4), *FAS* (relative mRNA level 562.9 ± 193.7), *TNFRSF1A* (relative mRNA level 458.2 ± 115.2), *TNFRSF1B* (relative mRNA level 62.9 ± 33.1) and *TNF* (relative mRNA level 44.1 ± 19.0). Active CASP3 protein could not be localized in the analyzed CL tissue (example of negative staining in Fig 6A). Strong staining for TNF protein was identified in non-steroidogenic cells, presumably macrophages (Fig 6D).

Discussion

Apoptosis has been shown to be highly involved in tissue remodeling during development and regression of the CL in a variety of species [8, 63], but no research has been done in the feline luteal phase. In our study, the mRNA of all factors analyzed was present in the CL tissue of domestic cat and lynx throughout all stages. We showed a significant change in the expression of *BCL2*, *CASP3*, *TNFRSFB1* and *FAS* throughout luteal stages in the domestic cat, what might indicate their potential involvement in the regulation of the feline luteal phase. The significantly elevated mRNA level of *TNFRSF1A* in both the domestic cat and the Iberian lynx during CL formation may suggest a possible conserved involvement of this factor in tissue reorganization and luteinization after ovulation. In contrast, the CL capacity to express *TNF* differed considerably between domestic cat and both Iberian and Eurasian lynx. The presence of mRNA of pro-apoptotic factors *BAX*, *CASP3*, *TNFRSFR1* and *FAS* in perCL of Iberian and Eurasian lynx suggests potential capacity of perCL to undergo apoptosis. Significantly higher expression of pro-survival factors *BCL2* and *TNFSFR1B* in perCL compared to frCL of Iberian lynx may be part of the mechanism to secure the structural integrity of perCL. Histologic analysis revealed a different structural state of perCL in Iberian and Eurasian lynx during the pre- and post-mating periods, respectively.

Gene expression of pro-apoptotic factors *BAX* and *CASP3* was found in CL throughout the whole luteal phase of the domestic cat. From the mRNA levels of *BAX* and *CASP3* analyzed in this study, it is not clear whether the protein product is activated; therefore, the data cannot indicate whether the apoptotic signal has been implemented. However, active CASP3 protein was detected in domestic cat luteal tissue by immunohistochemistry, showing local apoptotic processes in reorganization (early stages) and regression (late stages) of feline CL. Likewise, CL of other species express BAX and CASP3 at various stages [35, 36, 38, 47, 64–66]. Fas ligand could not be identified in the tissues studied and therefore was not included in the gene analysis. However, its receptor, *FAS*, was present in domestic cat CL and was constantly expressed throughout the non-pregnant luteal phase. In pregnancy, the regressing CL of the domestic cat showed higher expression of *FAS* compared to its previous stage, indicating a possible

involvement of the FAS/Fas ligand system in regulating feline luteolysis during pregnancy. Likewise, the increase in FAS and Fas ligand expression with regression of the CL has been shown in many species, including rodents and cattle [7, 42, 67].

In this study, luteal cells of the domestic cat, while presenting high receptivity to TNF *via* expression of *TNFRSF1A* and *TNFRSF1B*, were not identified as a source of this cytokine, in contrast to other species [43, 53, 68]. The feline TNF may be derived from other sources, such as immune and endothelial cells [43]. In both rat and bovine CL, TNFRSF1A has been associated with the processes of luteolysis [48, 51]. In our study, the relative mRNA level of *TNFRSF1A* was significantly higher during the CL formation stage in both the pregnant and non-pregnant luteal phases of the domestic cat. Moreover, such increased expression was also noted in the forming frCL of the Iberian lynx. We hypothesize that this receptor is involved in processes of CL formation and may be conserved for both domestic cat and Iberian lynx. The involvement of TNFRSF1A in CL formation can be as an apoptotic mediator of the tissue reorganization processes.

For the domestic cat, the process of luteolysis and differences in it between pregnancy and the non-pregnant luteal phase are not yet known. The CL of infertile cycles could regress in response to active luteolytic signals, like in sheep and rat [8, 63], or undergo a passive regression, like in dogs [69]. Based on our results, we hypothesize that the life span of CL of pregnancy is supported by luteotropic factors. The CL of non-pregnant luteal phases, therefore, may passively regress due to the absence of such support. These CL may still produce receptors potent for survival like in pregnancy (*TNFRSF1B* expression), but either lack the activation of the survival cascade by ligands or have the luteotropic signal blocked on the downstream level. This may also explain high variations in reported functional life spans of CL in non-pregnant luteal phases in domestic cats, *e.g.*, from 26 to 62 days as evidenced by serum progesterone levels [22, 23].

The histomorphology of Iberian lynx CL is described for the first time in this study. Due to the histomorphological similarity of Iberian lynx CL to the domestic cat, it was possible to distinguish between frCL undergoing formation and structurally maintained perCL by histologic analysis. Interestingly, perCL of Eurasian lynx before mating exhibited structural regression signs, while perCL of Iberian lynx after mating showed no signs of regression. Moreover, all of the perCL analyzed showed functional activity, as evidenced by intraluteal progesterone and estrogen concentrations. Histologic observations allow us to hypothesize that the state of perCL changes under the influence of new ovulations, returning it to the state of maintenance from regression. This is supported by reports on the bobcat, in which structurally persistent CL were responsive to gonadotropin treatment, *i.e.*, exhibited elevation in progesterone secretion [14].

In our study, we could only collect samples from two Iberian lynx, due to the critically endangered status of these species. The observations are, therefore, limited to the system in two animals; however, they allow us to partly answer introduced questions and establish the direction of further studies. The detection of pro-apoptotic factors *BAX*, *CASP3*, *TNFRSF1A* and *FAS* at the mRNA level in all CL analyzed suggests that perCL, although structurally persistent, have the capacity to undergo apoptosis. The next question would therefore be whether CL persist due to the absence of luteolytic signal or because such luteolytic signal is further blocked, supposedly *via* involvement of luteotropic factors. Based on our results, we suggest the second mechanism. Here, the candidate for the rescue factor is BCL2, which in higher concentrations would outcompete BAX for BH3-domain binding and thus either prevent BAX from activating or, if already activated, from proceeding to MOM permeabilization [70]. Additionally, TNFRSF1B may compete for the ligand binding with TNFRSF1A, resulting in the activation of pro-survival rather than pro-apoptotic pathways. Both these pro-survival factors, *BCL2* and

TNFRSF1B, were present at the mRNA level in perCL of Eurasian lynx and significantly higher expressed in perCL compared to frCL in analyzed Iberian lynx.

The phenomenon of physiological CL persistence is not common among mammalian species. So far, Eurasian, Iberian and Canada lynx are the only species where both structural and functional persistence of CL has been reported. In cows, the persistence of such functional CL is viewed as a pathology, because it prevents the initiation of a new ovarian cycle [71]. The unique physiological persistence of CL in lynx offers opportunity to learn more on the mechanisms of CL rescue. For instance, many human clinical studies are focused on a search for rescue factors to prevent premature regression of the CL. Physiologically, such rescue occurs during pregnancy, *e.g.*, in humans by chorionic gonadotropin [72, 73], or *post-partum* in lactating rats [74, 75]. In baboons, the administration of human chorionic gonadotropin or gonadotropin releasing hormone prolonged the life of CL even during the early follicular stage [76]. In all cases, rescue of the CL is accompanied by increased progesterone concentrations. Telleria suggests that regression of the CL can be interrupted and even reversed, either by rescuing its capacity to produce progesterone or by interfering with apoptosis [77]. As shown by previous studies [27] and by intraluteal hormone concentrations in our study, perCL of both Iberian and Eurasian lynx retain the capacity to produce progesterone. This characteristic could be the basis for the structural integrity of perCL and may involve potential luteal factors to ensure functional persistence. The elevation of pro-survival factors, such as *BCL2* and *TNFRSF1B*, could be a mechanism to prevent transduction of the apoptotic signal and rescue perCL from its structural demise. Interestingly, it is hypothesized that BCL2 is involved in prolongation of CL lifespan during pregnancy in humans [78]. Moreover, progesterone has been shown to promote BCL2 expression and decrease BAX to BCL2 ratio in bovine luteal cells [79]. It might be possible that the same mechanisms that rescue CL of the domestic cat during pregnancy, as discussed above, are involved in the physiological persistence of lynx CL. If this assumption is true, research on the luteotropic and luteolytic systems in the domestic cat becomes essential for understanding the unique reproduction of monoestrous lynx.

Interestingly, gene expression of the pro-apoptotic receptor *FAS* was significantly higher in maintained perCL compared to frCL of Iberian lynx. This observation leads to two possible scenarios: (i) either the elevated FAS indicates the increased susceptibility of perCL to apoptosis and with this the existence of a survival system to block the FAS-induced apoptotic signal, including the possible involvement of a soluble form of FAS [80]; or (ii) elevated FAS points to the pro-survival action of FAS itself. The latter is not unrealistic, because with the growing number of recent studies it is becoming clear that FAS can also exhibit diverse non-apoptotic actions depending on the tissue and conditions [81]. Consistent with this, elevated expression of FAS was found in early stage bovine CL compared to later stages [82]. In the second scenario, FAS might be one of the factors involved in the persistence of lynx CL and hence also one of the lynx reproductive characteristics divergent from those of the domestic cat. Another distinct characteristic of the lynx luteal system that was observed involves *TNF* gene expression. While mRNA levels of *TNF* were close to zero in the domestic cat CL, indicating an extra-ovarian source of this cytokine, in both Iberian and Eurasian lynx this factor was regularly expressed. Because the increased expression of *TNF* in perCL tissue coincided with a similar increase in *TNFRSF1B*, we suggest a local autocrine regulation of the perCL *via* this non-apoptotic receptor.

In summary, as in other species studied, apoptosis-related factors are expressed during different stages of luteal development and regression in the domestic cat CL. It appears that TNFRSF1A might play an important role during the formation of feline CL. In Iberian and Eurasian lynx, structurally intact perCL exhibited the potential capacity to undergo apoptosis. Of the factors studied here, we hypothesize that BCL2 and TNFRSF1B might play a luteotropic

role and be involved in the protection of perCL from complete structural regression. Mechanisms by which apoptosis-related factors are involved in the feline luteal phase are uncertain. Our current work presents a basis for further research on apoptosis system in the feline luteal phase and indicates potential factors involved in the regression and persistence of feline CL.

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Author Contributions

Conceived and designed the experiments: OA BCB KJ. Performed the experiments: OA LZ. Analyzed the data: OA. Contributed reagents/materials/analysis tools: JP RS FV. Wrote the paper: OA. Contributed to the discussion of the results: OA BCB KJ. Performed critical revision of the manuscript: LZ JP RS FV BCB KJ.

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4 CHAPTER III

Progesterone, estrogen and androgen receptors in the *corpus luteum* of the domestic cat (*Felis catus*), Iberian lynx (*Lynx pardinus*) and Eurasian lynx (*Lynx lynx*)

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Authors: Amelkina O, Zschockelt L, Painer J, Serra R, Villaespesa F, Krause E, Jewgenow K, Braun BC.

Contribution of each co-author to the manuscript:

OA carried out CL collection (cat) and histological analysis (cat and lynx), staged the samples based on their histomorphological appearance, performed RT-qPCR measurements, conducted statistical analysis, interpreted the data and wrote the article.

LZ contributed to molecular studies, i.e., RNA isolation

JP collected ovarian samples of Eurasian lynx, Norway

RS collected ovarian samples of the Iberian lynx, Portugal

FV collected ovarian samples of the Iberian lynx, Spain

EK performed mass spectrometry analysis

KJ participated in a study design and discussion of the results

BCB supervised the study and its design, carried out molecular studies, i.e., sequence analysis and primer design, RT-qPCR establishment, and participated in a discussion of the results

All co-authors revised the paper.

4.1 Abstract

In contrast to the species studied, *corpora lutea* (CL) of Iberian and Eurasian lynx physiologically persist in the ovary for more than two years and continue to secrete progesterone. Such persistent CL (perCL) transition into the next cycle and are present in the ovary together with the freshly ovulated CL (frCL). To date, the mechanisms supporting such luteal persistence are not known. Progesterone, estrogens and androgens play an essential role in mammalian reproduction, including regulation of CL formation, maintenance and regression. We analyzed the potential receptivity of feline CL to these steroids *via* mRNA measurements of nuclear progesterone receptor (PGR), progesterone receptor membrane components (PGRMC) 1 and 2, nuclear estrogen receptors (ESR) 1 and 2, G protein-coupled estrogen receptor 1 (GPER1) and androgen receptor (AR). All receptors were present in domestic cat CL during pregnancy and non-pregnant luteal phase, in frCL and perCL of post-mating Iberian lynx and in perCL of pre-mating Eurasian lynx. Mass spectrometry detected the presence of PGRMC1 protein in frCL and perCL of the Iberian lynx. The mRNA levels of *PGR*, *PGRMC1*, *PGRMC2*, *ESR1* and *AR* changed significantly throughout the domestic cat luteal phase. This may indicate involvement of these receptors in the processes of formation, maintenance and regression of feline CL. In Iberian lynx, perCL differed from frCL in significantly higher mRNA expression of *PGRMC1*, *PGRCM2*, *ESR1*, *GPER1* and *AR*. Only for *ESR2*, the expression was reversed. High mRNA amounts of these receptors in perCL suggest that physiological persistence of lynx CL may be partly regulated by actions of progesterone, estrogens and androgens *via* their nuclear and membrane receptors.

4.2 Introduction

Reproductive patterns vary widely among species and are heavily dependent on the function of the *corpus luteum* (CL), a transient gland that forms in the ovary after ovulation and maintains pregnancy in many species *via* its production of progesterone (Niswender et al.

2000). In dogs, CL are the only source of progesterone (Hoffmann et al. 2004), while in sheep the placenta takes over the production of this steroid during mid-pregnancy (Al-Gubory et al. 1999). Multiple accessory CL are formed in the ovaries of elephants from non-ruptured follicles to support their extremely long pregnancy with additional sources of progestagens (Hildebrandt et al. 2011). In the domestic cat (*Felis catus*), CL are the main source of progesterone, and the placenta is a supplemental site of synthesis (Braun et al. 2012b, Siemieniuch et al. 2012, Verstegen et al. 1993). In all mammals studied so far, including the domestic cat, CL regress from ovarian tissue at the end of pregnancy or in the non-pregnant luteal phase, allowing the initiation of a new cycle. The situation, however, is markedly different for another member of the *Felidae* family, the lynx. Studies on the *Lynx* genus have revealed that Iberian and Eurasian lynx (*Lynx pardinus* and *Lynx lynx*, respectively) exhibit a non cat-like ovarian cycle, in which CL physiologically persist in the ovary for more than two years and remain functionally active in their production of steroids (Carnaby et al. 2012, Goritz et al. 2009, Jewgenow et al. 2014, Painer et al. 2014). The mechanisms underlying such physiological persistence of CL are not clear, and studies to unravel this reproductive peculiarity are only just beginning.

The domestic cat has been considered as seasonally polyestrous (the length of breeding season decreases with increasing latitudes) and as an induced ovulator (Freistedt et al. 2001, Hurni 1981, Shille et al. 1983, Wildt et al. 1981); however, spontaneous ovulation without cervical stimulation also occurs (Gudermuth et al. 1997, Pelican et al. 2005). After ovulation, queens can either enter a period of pregnancy (approximately 65 days (Tsutsui and Stabenfeldt 1993)) or a non-pregnant luteal phase (approximately 40 days (Wildt et al. 1981)). In both scenarios, elevated serum progesterone decreases towards the end of the luteal phase, allowing initiation of a subsequent ovarian cycle (Shille and Stabenfeldt 1979, Verhage et al. 1976). The intraluteal concentration of progesterone and the capacity of CL to produce it also decline by the end of pregnancy and the non-pregnant luteal phase (Amelkina et al. 2015, Zschockelt et al. 2014). Changes in estrogen and progesterone concentrations throughout the estrous cycle of the

domestic cat have been studied intensively and include observations on serum, metabolite and tissue levels (Braun et al. 2012b, Brown et al. 2001, Paape et al. 1975, Roth et al. 1997, Shille and Stabenfeldt 1979, Stewart et al. 2012, Swanson et al. 1995, Tsutsui et al. 2009, Verhage et al. 1976). Interestingly, at least in two independent studies, CL were found on the ovary of lactating domestic cats 63 days after parturition (Amelkina et al. 2015, Dawson 1946).

The *Lynx* genus includes four species: the Eurasian lynx, the Iberian lynx, the Canada lynx (*Lynx canadensis*) and the bobcat (*Lynx rufus*). In contrast to the domestic cat, all lynx species, excluding the bobcat, are identified as monoestrous (Jewgenow et al. 2014). In the three monoestrous species, functional activity of persistent CL (perCL) was confirmed outside of pregnancy and weaning (Carnaby et al. 2012, Fanson et al. 2010, Goritz et al. 2009, Painer et al. 2014). Moreover, in our recent study perCL of Iberian and Eurasian lynx exhibited steroidogenic activity (Zschockelt et al. 2015). In bobcats, CL persist morphologically, yet their functional activity has not been investigated (Crowe 1975, Duke 1949). We hypothesize that one of the roles of perCL in lynx (except the bobcats) is to ensure a monoestrous cycle *via* suppression of ovarian activity, which would ensure the birth and weaning of cubs during the most favorable time of the year.

The steroid hormones progesterone, estrogens and androgens play an essential role in female reproduction and initiate and maintain a wide variety of processes, including folliculogenesis, ovulation, implantation, decidualization and parturition (Gellersen et al. 2009, Peluso 2006, Rosenfeld et al. 2001, Soltysik and Czekaj 2013, Stocco et al. 2007). Formation, maintenance and regression of the CL fall under the influence of these hormones as well, and CL themselves have the capacity to express steroid receptors (Stocco et al. 2007). Progesterone actions can be transduced in the CL *via* its nuclear receptor PGR, membrane progestin receptors (mPRs) and progesterone membrane components (PGRMC) 1 and 2 (Kowalik et al. 2013a). Estrogen has at least two nuclear receptors, ESR1 and ESR2 (also known as estrogen receptor alpha and beta, respectively), the yet to be determined membrane receptors (mERs) and the recently discovered

G protein-coupled estrogen receptor 1 (GPER1) (Bottner et al. 2014, Maggiolini and Picard 2010, Rosenfeld et al. 2001, Soltysik and Czekaj 2013). Finally, the androgen receptor (AR) has been identified in ovarian tissue of many species (Hampton et al. 2004, Horie et al. 1992, Slomczynska et al. 2006, Weil et al. 1998).

The PGR, ESR1, ESR2 and AR belong to the nuclear receptor superfamily and act as hormone-dependent transcriptional factors. After binding to the ligand and subsequent release from heat shock proteins, receptors undergo translocation to the nucleus and dimerization, and with the recruitment of co-factors they regulate gene transcription on specific sequences of DNA (Griekspoor et al. 2007). Such signal transduction *via* nuclear receptors is called the classical genomic pathway, and the resultant effect can be delayed for hours or even days. Additionally, a non-genomic pathway of rapid signaling exists *via* membrane receptors (such as PGRMC1, PGRCM2 and GPER1).

To our best knowledge, there is yet no information on the receptivity to steroid hormones of the feline CL, including the model felid, the domestic cat. To fill this gap, we focused on the detection of mRNA for nuclear and membrane receptors of progesterone (PGR, PGRMC1 and PGRMC2), estrogen (ESR1, ESR2 and GPER1) and androgen (AR) in the CL tissue of the domestic cat, as well as on the changes in its levels throughout pregnancy and the non-pregnant luteal phase. Moreover, we analyzed the relative mRNA amounts of these receptors in the perCL of Iberian and Eurasian lynx and in the CL of fresh ovulation (frCL) of the Iberian lynx to learn more about the uncommon features of lynx reproduction. The knowledge obtained here could contribute to future studies on feline reproduction, assist in the development of reproductive techniques for the Iberian lynx (critically endangered species; Palomares et al. 2011) and advance our understanding of the physiology of CL persistence in general.

4.3 Materials and Methods

All chemicals in the study were purchased from Sigma–Aldrich (Taufkirchen, Germany), unless stated otherwise and were of the highest purity available. The methods applied, and the study design, were approved by the Internal Committee for Ethics and Animal Welfare of the Leibniz Institute for Zoo and Wildlife Research in Berlin, Germany (Permit numbers: 2010-10-01 and 2011-01-01).

4.3.1 Animals and tissue collection

Ovaries of domestic cats were obtained from local animal shelters and clinics after ovariohysterectomy for the purpose of permanent contraception. Samples were transported in MEM-HEPES medium, supplemented with 3 mg/mL BSA (Merck Millipore, Darmstadt, Germany) and 1x Antibiotic Antimycotic Solution. Transportation was at 4 C, and ovaries were processed immediately after arrival at the laboratory (2-4 h after surgery). The isolation and consequent staging of CL is described in Amelkina *et al.* (Amelkina et al. 2015). In brief, CL from each cat were either fixed in Bouin's solution for histological analysis or plunged into liquid nitrogen for RNA isolation. In the case of pregnancy, the day was assessed by the diameter of the gestation chamber (Zambelli and Prati 2006), the crown-rump length of a fetus (Schnorr B 2006) or by the stage of pre-implantation embryos (Knospe 2002). The pre-implantation period (n = 6) included samples from days 2 to 6 and 10 *post-coitum*; the post-implantation period (n = 11) included samples from days 14 to 36; finally, the CL regression stage (n = 5) was represented by samples from days 38, 39 and 48 and week 9. The absence of embryos in the oviducts or uteri indicated a non-pregnant luteal phase. In such cases, based on their histologic appearance, each CL was classified as the stage of: formation (n = 9), development/maintenance (n = 13), early regression (n = 14), late regression (10) or *corpus albicans* (CA; n = 4). The histologic classification is described in detail in Amelkina *et al.* (Amelkina et al. 2015) and includes parameters of: cell shape, type and degree of vacuolation,

nucleus condition, and the ratio of non-steroidogenic to luteal cells. Listed n-values represent the number of animals per analyzed stage; each animal is represented by one CL.

Ovaries of Iberian lynx were collected within the scope of the Iberian lynx conservation breeding program at the Centro de Cría de Lince Ibérico El Acebuche, Parque Nacional de Doñana, Huelva, Spain and the Centro Nacional de Reprodução do Lince Ibérico, Silves, Portugal. Two animals were ovariohysterectomized for medical reasons, seven days after ovulation was induced by natural mating in February, 2013. In one animal (Iberian lynx 1, nine years old), embryos were flushed from the uterus, indicating the pre-implantation stage of pregnancy. Unfertilized oocytes were flushed from the second animal (Iberian lynx 2, eleven years old), thus indicating a non-pregnant luteal phase. The CL were isolated immediately after surgery and their morphological appearance was noted. The presence of ovulation scars distinguished CL of fresh ovulation (frCL) from CL of previous cycle/s (perCL). Each type of CL was collected with the following sample sizes: Iberian lynx 1, n = 3 for frCL and n = 8 for perCL; Iberian lynx 2, n = 5 for frCL and n = 6 for perCL. Each CL was dissected and pieces were placed in RNA-later solution (Qiagen GmbH, Hilden, Germany) for RNA isolation.

Ovaries of free-ranging Eurasian lynx were collected freshly *post-mortem* from animals hunted legally during the national hunting quota for management purposes in Norway (n = 5 animals). The period of collection was the beginning of the breeding season prior to mating (February, 2011; the breeding season for the Eurasian lynx in Norway is February to early April (Kvam 1991)). After dissection, samples were immediately placed in RNA-later for RNA isolation. Based on the pre-breeding period of collection and the absence of frCL, embryos and placental scars of a recent pregnancy, all isolated CL were classified as perCL from previous cycle/s.

4.3.2 RNA isolation and cDNA synthesis

Up to 26 mg of CL tissue was homogenized in homogenization tubes (100 µl RNA lysis buffer, 1.4/2.8 mm ceramic beads) at 5000 rpm for 2 x 25 sec (Precellys 24 homogenizer, Bertin Technologies, Montigny-le-Bretonneux, France). Total RNA was extracted using the innuSPEED Tissue RNA/innuPREP DNase I Digest Kit (Analytik Jena AG, Jena, Germany). The NanoDrop ND-1000 (PEQLAB Biotechnologie GmbH, Erlangen, Germany) was used to assess the concentration and purity of isolated RNA. Additional control of RNA quality and integrity was performed *via* microfluidic analysis using the Bioanalyzer (Agilent Technologies Deutschland GmbH, Boeblingen, Germany); RNA integrity number (RIN) values were above 7.0 for cat samples and above 6.1 for Iberian and Eurasian lynx samples. From 1 to 2.5 µg of isolated RNA was reverse-transcribed into single-stranded (ss) cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Biosciences GmbH, St. Leon-Rot, Germany). Reverse transcriptase was substituted with RNAse-free water in the negative control to verify the absence of genomic DNA contamination.

4.3.3 Sequencing

Primers for the polymerase chain reaction (PCR) were purchased from BioTeZ Berlin Buch GmbH (Berlin, Germany) and were designed based on predicted (*PGR, PGRMC1, PGRMC2, ESR2, GPER1, AR*) or published (*ESR1*) *Felis catus* gene sequences. Primer information is listed in Table 1. Based on feline ss cDNA templates of ovary, uterus, oviduct or placenta origin, partial cat and lynx cDNA sequences were amplified using the Expand High FidelityPLUS PCR system (Roche Diagnostics Deutschland GmbH, Mannheim, Germany), as described by Braun *et al.* (Braun et al. 2012a). For both cat and lynx, the PCR conditions were: 94 °C for 2 min; followed by 35 cycles of denaturation at 94 °C for 30-60 sec, annealing at 49-53 °C (see Table 1) for 30-60 sec, elongation at 72 °C for 60-160 sec; and final elongation at 72 °C for 7 min. Purified PCR products were ligated to the pCR4-TOPO TA vector and transfected into one shot

TOPO10 cells (both Life Technologies GmbH, Darmstadt, Germany) for *AR* or DH5 alpha cells (Life Technologies GmbH) for the remaining genes. Positive clones were sequenced by the Services in Molecular Biology GmbH (Dr M. Meixner, Rüdersdorf, Germany). For some genes (*PGRMC1, PGRMC2* and *ESR2*), only lynx-specific PCR products were sequenced, but not cloned.

4.3.4 Quantitative PCR

Primers for quantitative real-time PCR (qPCR) were designed based on cat and lynx sequences identified in this study (Table 1). The qPCR was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories GmbH, Munich, Germany), as published by Braun *et al.* (Braun et al. 2012b). In brief, diluted ss cDNA (4 µl, corresponding to 2 or 10 ng of total RNA for genes of interest, or 4 ng for reference genes) were analyzed in a 10 µl reaction volume including SsoFast EvaGreen Supermix (Bio-Rad Laboratories GmbH) and primers (Table 1). The qPCR conditions were: 98 °C for 2 min and 40 cycles of 8 sec at 98 °C and 8 sec at different annealing temperatures (Table 1). Quantification of qPCR products was performed using the CFX Manager Software 1.6 (Bio-Rad Laboratories GmbH). Serial dilutions of plasmid DNA carrying genes of interest sequences were used for calibration. Glutaminase (*GLS*; for domestic cat, JQ424891), TATA box binding protein (*TBP*; for domestic cat, JQ424890; for lynx, JX993351), β-actin (*BACT*; for domestic cat, AB051104; for lynx, KM458620), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH;* for lynx, KM458621) and ribosomal protein S7 (*RPS7*; for lynx, JX993349) were validated as optimal reference genes in the feline CL with the qbasePLUS software (Biogazelle, Zwijnaarde, Belgium; (Hellemans et al. 2007)) and were used for normalization. A multiple normalization factor was calculated for individual CL referring to Vandesompele *et al*. (Vandesompele et al. 2002).

Table 1
Sequences of primers used for sequence analysis and expression studies, annealing temperatures, and product sizes.

Factor	GenBank ID	Species	Primer sequence 5' – 3'	T_A (°C)	Product size (bp)	Use
PGR	KF831338*	*Felis catus*	*PGR* Fw: GCA TGT CGC CTT AGA AAG TG	53.0	903	a
	KF831339*	*Lynx lynx*	*PGR* Rv: CAA GAT CTT GGG TAA CTG TG			
			PGR qFw: GTG GCA GAT TCC ACA GGA GT	60.0	179	b
			PGR qRv: TTT GCC TCA GAC CAA TTG C			
PGRMC1	KF831340*	*Felis catus*	*PGRMC1* Fw: CCA ACC TTT GCT CCA GMG A	Fe. 53.0	666	a
	KF831341*	*Lynx pardinus*	*PGRMC1* Rv: GGA ATG TTA CAA ATG ATT CTG C	Ly. 51.0		
			PGRMC1 qFw: AGC CCT GAA GGA CGA GTA TG	59.0	176	b
			PGRMC1 qRv: GAG CGC TCT CAT CTT TTG CT			
PGRMC2	KF831342*	*Felis catus*	Fe.*PGRMC2* Fw: GAC GTG AAG CTA GGC ACC	53.0	679	a
	KF831343*	*Lynx pardinus*	Fe.*PGRMC2* Rv: CCC TGA CTT TGG TTG TTT AC			
			Ly.*PGRMC2* Fw: GGA CTT CAG GGA GCA G	51.0	395	a
			Ly.*PGRMC2* Rv: CCC TGA CTT TGG TTG TTT AC			
			PGRMC2 qFw: TCG CGG TCA ATG GGA AAG T	62.5	112	b
			PGRMC2 qRv: AAT GTC GCC AGT CCT CTG GA			
ESR1	AY462090.1	*Felis catus*	Ly.*ESR1* Fw: CTC AAC AGC GTG TCT CCG AG	51.0	1111	a
	KF831330*	*Lynx lynx*	Ly.*ESR1* Rv: TCC AGA GAC TTC AGG GTG CT			
			ESR1 qFw: CCA TGG AGT CTG CCA AGG	59.5	158	b
			ESR1 qRv: GCA CTG GTT GGT AGC TGG A			
ESR2	KF831331*	*Felis catus*	Fe.*ESR2* Fw: GAG TTT CCT CAG CTG TTA CC	53.0	1520	a
	KP826768*	*Lynx lynx*	Fe.*ESR2* Rv: GCA TTC AGC ATC TCC AGC A			
			Ly.*ESR2* Fw: GAG TTT CCT CAG CTG TTA CC	51.0	524	a
			Ly.*ESR2* Rv: GAC CAG ACT CCA TAG TGA TA			
			ESR2 qFw: TAG CGA TCC ATT GCC AGT C	59.0	134	b
			ESR2 qRv: CAC AAC TGC TCC CAC TAA CC			
GPER1	KF831333*	*Felis catus*	*GPER1* Fw: AGT ACT TCA TCA ACC TGG C	Fe. 53.0	556	a
	KF831334*	*Lynx lynx*	*GPER1* Rv: ACR CTG ATG AAS ACR TTC TC	Ly. 49.0		
			GPER1 qFw: GTC ACC CTG GGC TTC ATC	61.5	131	b
			GPER1 qRv: AAG ATC ATG CGG AGG GCT T			
AR	KM368272*	*Felis catus*	*AR* Fw1: ATG GAG GTR CAG TTA GGG YT	53.0	1327[1]	a
	KM368273*	*Lynx lynx*	*AR* Rv1: CTG TGA ASA GAG TRT GCC AG			
			AR Fw2: CAA GCT GGA RAA CCC DCT G	53.0	1575[1]	a
			AR Rv2: GGG TYT CYA AAG CTT CAC TG			
			AR qFw: GGA ACT TGA TCG TAT CAT TGC	56.5	176	b
			AR qRv: CAT TTC TGG AAA GTC CAC G			

continuation of the Table 1
bp, base pair; Fw, forward; Rv, reverse; T_A, annealing temperature; a, used for sequence analysis; b, used for gene expression studies
Fe., *Felis catus*; Ly., *Lynx lynx/pardinus* – indicate differences between analysis of cat and lynx tissue
[1] final length of a consensus sequence without primers is 2701 bp
* GenBank sequences were obtained in this study

4.3.5 Protein identification by mass spectrometry

Lysates of two Iberian lynx CL were separated by SDS-PAGE. Lanes were cut into 10 equal-sized bands, and protein digestion was performed as previously described (Lange et al. 2010). Briefly, gel bands were incubated with 50 ng trypsin (Promega, Madison, WI, USA) in 15 µl 50 mM ammonium bicarbonate buffer overnight at 37 °C. Ten µl of 0.5% TFA in acetonitrile was added before drying the samples under vacuum. Samples were resuspended in 6 µl 0.1% (v/v) TFA and 5% (v/v) acetonitrile. Peptides were analyzed by a reversed-phase capillary liquid chromatography system (Ultimate 3000 nanoLC system, Thermo Fisher Scientific) connected to an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). LC separations were performed on a capillary column (Acclaim PepMap100, C18, 3 µm, 100 Å, 75 µm i.d. × 25 cm, Thermo Fisher Scientific) at an eluent flow rate of 300 nL/min using a linear gradient of 3–25% B in 60 min with further increase to 80% B in 80 min. Mobile phase A contained 0.1% formic acid in water, and mobile phase B contained 0.1% formic acid in acetonitrile. Mass spectra were acquired in a data-dependent mode with one MS survey scan with a resolution of 60,000 (Orbitrap Elite) and MS/MS scans of the 15 most intense precursor ions in the linear trap quadrupole.

Protein identification was performed using MaxQuant (version 1.4.1.1, Martinsried, Germany) software. Data were searched against the Uniprot protein database (December 2014) or a self-made database that contained the (partly predicted) *Felis catus* sequences of the receptors (XP_011284806.1, XP_011284807.1, XP_003985064.1, XP_003998435.1, NP_001019402.1, XP_003987774.1), one Panthera tigris sequence (XP_007089200.1) and

lynx and cat protein information derived from the DNA receptor sequences analyzed in this study. The criteria for identification were set to at least 1 unique peptide and at least 2 razor + unique peptides.

4.3.6 Statistical analysis

Statistical analysis was performed with the R software package (R: A language and environment for statistical computing, version 3.0.0, Vienna, Austria). For the domestic cat, the Kruskal-Wallis rank sum test was used to determine changes in relative mRNA amounts throughout pregnant and non-pregnant luteal phases. The Wilcoxon rank sum test was used for *post-hoc* pairwise comparison of stages (P-value adjustment: Benjamini-Hochberg). Each animal was represented by one CL. Numbers of animals per stage can be found above in 2.2. The stage of CA was not included in the statistical analysis, because its origin (pregnant or non-pregnant luteal phase) in the ovary was unknown. For the Iberian lynx, the Mann-Whitney U-test was used to determine changes in relative mRNA amounts between frCL and perCL in each animal. Numbers of CL per stage per animal can be found above in 2.2. Probability (P) values less than 0.05 were considered statistically significant. SigmaPlot 10.0 (Systat Software Inc., San Jose CA, USA) was used to visualize the statistical results *via* boxplots.

4.4 Results

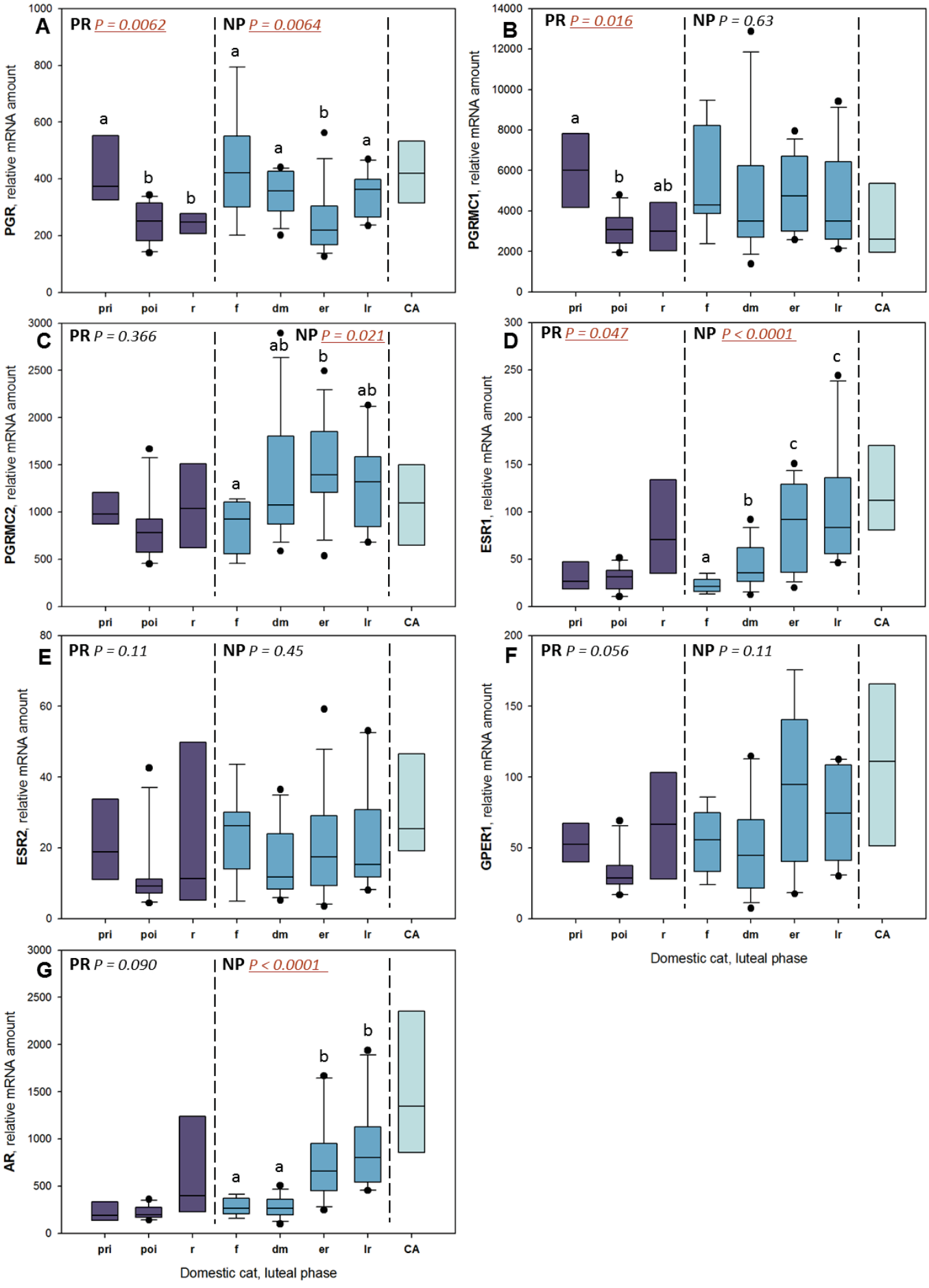
4.4.1 Feline gene sequences of steroid receptors

In this study, we identified partial cat and lynx gene sequences for the factors analyzed: *PGR*, *PGRMC1*, *PGRMC2*, *ESR1* (only lynx), *ESR2*, *GPER1*, *AR* (Table 1). The consensus sequence of *AR* varied in CAG-rich sites between *Felis catus* and *Lynx lynx*. All other partial sequences varied in single nucleotide substitutions between cat and lynx, not affecting the amino acid sequence.

4.4.2. Domestic cat

The mRNA of all receptors studied was present in CL tissue of all stages in the domestic cat. In the pre-implantation stage of pregnancy, mRNA amounts were higher for *PGR* (Fig. 1A) compared to both post-implantation (P = 0.0065) and regression (P = 0.0065) stages and for *PGRMC1* (Fig. 1B) compared to the post-implantation stage (P = 0.0092). Amounts of *ESR1* mRNA changed significantly throughout pregnancy (Fig. 1D); the subsequent *post-hoc* test showed a tendency for higher expression in regression compared to the post-implantation stage (P = 0.058). There was a tendency for change of mRNA amount for *GPER1* (Kruskal-Wallis test; Fig. 1F).

In the non-pregnant luteal phase, mRNA amounts of *PGR* (Fig. 1A) changed significantly throughout the CL stages and were the lowest in early regression compared to formation (P = 0.021), development/maintenance (P = 0.021) and late regression (P = 0.026). The amount of *PGRMC2* (Fig. 1C) mRNA was higher in early regression compared to the formation stage (P = 0.0039). The changes in *ESR1* (Fig. 1D) mRNA amount throughout the luteal stages were highly significant: it increased in development/maintenance compared to formation (P = 0.016), and in early regression compared to formation (P = 0.00033) and development/maintenance (P = 0.016), and in late regression compared to formation (P = 0.00013) and development/maintenance (P = 0.0039). High significance was also observed in the change of mRNA amount of *AR* (Fig. 1G) throughout the non-pregnant luteal phase: it was higher in early regression compared to formation (P = 0.00051) and development/maintenance (P = 0.00051), and in late regression compared to formation (P = 0.00013) and development/maintenance (P = 0.00032).


A
PR P = 0.0062
NP P = 0.0064
PGR, relative mRNA amount
B
PR P = 0.016
NP P = 0.63
PGRMC1, relative mRNA amount
C
PR P = 0.366
NP P = 0.021
PGRMC2, relative mRNA amount
D
PR P = 0.047
NP P < 0.0001
ESR1, relative mRNA amount
E
PR P = 0.11
NP P = 0.45
ESR2, relative mRNA amount
F
PR P = 0.056
NP P = 0.11
GPER1, relative mRNA amount
G
PR P = 0.090
NP P < 0.0001
AR, relative mRNA amount
pri poi r f dm er lr CA
Domestic cat, luteal phase

continuation of the Figure 1

luteal phases in the domestic cat. Box plots depict the distribution of data; whiskers indicate maximum and minimum values; black dots indicate outliers; horizontal lines indicate the median; P-values are calculated from the Kruskal-Wallis rank sum test; letters (a, b) identify significant differences between stages and are calculated from *post hoc* pairwise comparisons (P-value adjustment: Benjamini-Hochberg). PR: pregnancy; PP: non-pregnant luteal phase; pri: pre-implantation period; poi: post-implantation period; r: regression; f: formation; d/m: development/maintenance; er: early regression; lr: late regression; CA: *corpus albicans*.

4.4.3. Iberian and Eurasian lynx

The mRNA of all factors studied was present in all types of CL examined in Iberian and Eurasian lynx. The highest mRNA amount was observed for *PGRMC1* in both lynx species, being at least 10-fold higher than the rest of the factors examined.

In the Iberian lynx, no significant changes were observed in *PGR* (Fig. 2A) expression. Relative mRNA amounts of *PGRMC1* (Fig. 2B) were higher in perCL compared to frCL of Iberian lynx 1 ($P = 0.012$) and Iberian lynx 2 ($P = 0.0043$). Likewise, relative mRNA amounts of *PGRMC2* (Fig. 2C) were higher in perCL compared to frCL of Iberian lynx 1 ($P = 0.012$) and Iberian lynx 2 ($P = 0.0043$). For *ESR1* (Fig. 2D) and *GPER1* (Fig. 2F), relative mRNA amounts were higher in perCL compared to frCL of Iberian lynx 1 (both $P = 0.012$). The expression of *ESR2* (Fig. 2E) was reversed and was higher in frCL compared to perCL of Iberian lynx 1 ($P = 0.048$) and Iberian lynx 2 ($P = 0.0043$). Finally, relative mRNA amounts of *AR* (Fig. 2G) were higher in perCL compared to frCL of Iberian lynx 1 ($P = 0.012$).

In pre-mating CL of Eurasian lynx, the amounts of mRNA determined for all factors analyzed were within the range of post-mating CL of Iberian lynx. Expression levels of *PGRMC1* (relative mRNA amount per ng total RNA 4288.7 ± 2376.4) and *PGRMC2* (relative mRNA amount 589.9 ± 115.9) in perCL of pre-mating Eurasian lynx were closer to the range of fresh CL, rather than the persistent CL of post-mating Iberian lynx. In contrast, expression levels of

ESR2 (relative mRNA amount 6.9 ± 3.3) in perCL of pre-mating Eurasian lynx fell closer to the range of perCL of the post-mating Iberian lynx. The rest of the studied genes had the following expression levels: *PGR* (relative mRNA amount 159.1 ± 69.4), *ESR1* (relative mRNA amount 80.1 ± 30.3), *GPER1* (relative mRNA amount 77.8 ± 13.9) and *AR* (relative mRNA amount 198.3 ± 78.5).

The mass spectrometry analysis identified protein of PGRMC1 in the lysates of both frCL and perCL in the Iberian lynx (Table 2).

Table 2
PGRMC1 peptides identified with mass spectrometry and their localization within the amino acid (aa) sequence

peptide	aa position	peptide sequence
1	71-79	RDFTPAELR
2	72-79	DFTPAELR
3	72-80	DFTPAELRR
4	80-88	RFDGVQDPR
5	81-88	FDGVQDPR
6	105-119	KFYGPEGPYGVFAGR
7	106-119	FYGPEGPYGVFAGR
8	173-192	EGEEPTVYSDEEEAKDESAR

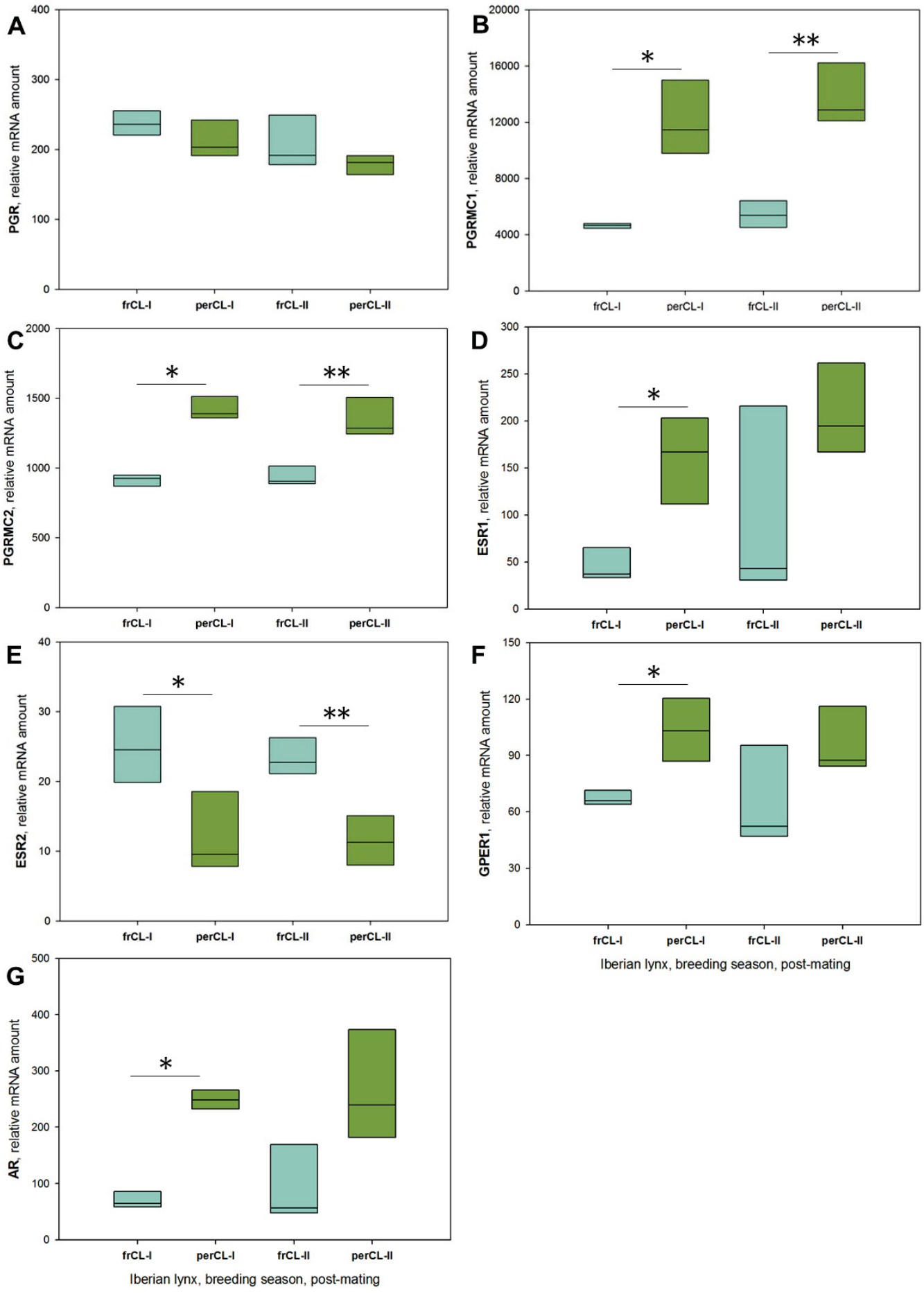


Figure 2 Intraluteal relative mRNA amounts per ng of total RNA during the breeding season in

continuation of the Figure 2

post-mating Iberian lynx. Box plots depict the distribution of data; horizontal lines indicate the median. frCL-I/II: frCL from Iberian lynx 1/2; perCL-I/II: perCL from Iberian lynx 1/2 * $P < 0.05$, ** $P < 0.005$; Mann-Whitney U-test

4.5 Discussion

Research into the mechanisms of steroid action *via* its receptors is challenging. Not only might there exist as yet uncharacterized receptors and their isoforms, but also the involvement of co-factors, such as co-activators and co-inhibitors on the stage of transcription mediation, can shift the initial signal of a ligand (Jamnongjit and Hammes 2006, O'Malley 2005). Such studies are further hindered when investigating steroid pathways in wild animals due to the limited access to samples for collection. In this regard, the collection of ovarian tissue from the Iberian lynx was complicated due to the critically endangered status of this species and was only possible for two ovariohysterectomized animals. For the Eurasian lynx, sample collection occurs only occasionally in the context of hunting or carcass harvesting; this collection is further complicated by the restricted time period authorized for hunting and because molecular analysis is mostly inapplicable due to the tissue condition of harvested carcasses. Finally, species-specificity is a limiting factor for functional studies. Even for the domestic cat, the information on the factors studied is scarce, and the number of suitable antibodies for proteins of interest is extremely low. As an initial stage in research on steroid receptor pathways of the feline CL, we investigated the potential receptivity of luteal tissue to steroids in the domestic cat and two lynx species, Iberian and Eurasian. Previously, we demonstrated the steroidogenic capacity of the same CL in domestic cats (Zschockelt et al. 2014) and Iberian and Eurasian lynx (Zschockelt et al. 2015).

In our study, we demonstrated that the feline CL has the capacity to express nuclear and membrane receptors of progesterone (PGR, PGRMC1, PGRMC2), estrogen (ESR1, ESR2,

GPER1) and androgen (AR). The mRNA of these receptors was identified in CL of domestic cats at all stages of pregnancy and in the non-pregnant luteal phase, as well as in frCL and perCL of post-mating Iberian lynx and perCL of pre-mating Eurasian lynx. The intraluteal expression of *PGR*, *PGRMC1*, *PGRMC2*, *ESR1* and *AR* changed significantly throughout the domestic cat luteal phase. In Iberian lynx, perCL differed from frCL in significantly higher expression of *PGRMC1*, *PGRCM2*, *ESR1*, *GPER1* and *AR*. For *ESR2* only, expression in perCL was significantly lower than in frCL. Such changes in mRNA amounts depending on the CL stage might indicate an important role of the steroid receptors analyzed here in the feline luteal phase. Below, we discuss possible roles of sex steroid receptors in the formation, maintenance and regression of the CL in domestic cats, as well as potential involvement of these receptors in the mechanism of physiological CL persistence in lynx.

Progesterone exerts a number of effects in the ovary, including mediation of follicular growth, ovulation, luteinization and estrogen secretion, as well as its own secretion in the CL (Gellersen et al. 2009, Peluso 2006, Stouffer 2003). Transduction of the multiplicity of progesterone actions is implemented *via* its receptors in reproductive tissues (Mulac-Jericevic and Conneely 2004). A mouse model in which the *Pgr* gene was knocked out showed that nuclear receptivity for progesterone played a key role in the process of follicle rupture (Lydon et al. 1996). In our study, the mRNA amount of *PGR* during formation of the CL was the highest in pregnancy and was higher compared to early regression in the non-pregnant luteal phase, which might indicate a specific role of progesterone during the early CL stage in the domestic cat. For instance, progesterone regulates mitosis and apoptosis of granulosa cells in rats (Svensson et al. 2000), and has anti-apoptotic effects in bovine luteal cells (Liszewska et al. 2005, Rueda et al. 2000).

The literature distinguishes two isoforms of PGR, PGRA and PGRB, both transcribed from the same gene under the influence of two different promoters (Kowalik et al. 2013a). Importance of the contribution of each isoform has been demonstrated on the knock-out mice models, where ablation of PGRA led to female infertility (Conneely 2010). The expression and

ratio of these isoforms has been studied in the CL of cows, (Rekawiecki et al. 2008), monkeys (Duffy et al. 1997) and humans (Misao et al. 1998). There is no information on PGR isoforms in feline CL tissue, and in our study we analyzed all mRNA variants of the *PGR* gene that contains exons 6 and 7, based on the qPCR primers used here. A future study is planned to analyze potential variants of PGR mRNA and protein in the domestic cat in more detail, which would allow investigation of changes in the ratio of these isoforms throughout the feline luteal phase.

The receptivity of CL to steroids is species-specific, and not all steroid receptors are expressed at every stage of the ovarian cycle. For example, no PGR mRNA and protein are detected in the rat CL (Goyeneche et al. 2003, Telleria et al. 1999); however, the mRNA of several membrane receptors, including PGRMC1, has been measured during pregnancy (Cai and Stocco 2005). In our study, in contrast to the domestic cat *PGR* expression profile, we did not observe any differences in the expression of *PGR* between frCL and perCL of Iberian lynx. In contrast, the mRNA amounts of *PGRMC1* and *PGRMC2* changed significantly and were higher in perCL compared to frCL of both Iberian lynx. Moreover, we clearly identified PGRMC1 protein in both frCL and perCL of the Iberian lynx via mass spectrometry. This conforms to the results on mRNA level, where the expression of *PGRMC1* was at least 10 fold higher than the rest of analyzed factors. Together our findings indicate that progesterone action in the lynx might be regulated mainly *via* membrane receptors, particularly PGRMC1, and thus play a supporting role in CL persistence. Indeed, the expression of PGRMC1 in the bovine CL is dependent on the stage of the estrous cycle, and it was suggested that PGRMC1 promotes luteal cell viability by mediating the anti-apoptotic action of progesterone (Luciano et al. 2011). Moreover, our recent histologic observations of the CL analyzed herein suggest that the structural and presumably functional state of perCL changes under the influence of new ovulations, returning to the stage of maintenance (perCL of post-mating Iberian lynx) from early regression (perCL of pre-mating Eurasian lynx; Amelkina et al 2015, under revision). It

is possible that the rescuing action of progesterone on perCL in lynx is regulated *via PGRMC1*, rather than *PGR*. Freshly formed CL of the new ovulation may be then an initial source of progesterone.

The expression of PGRMC1 mRNA has been identified in granulosa and/or luteal cells of cows (Kowalik and Kotwica 2008), pigs (Jiang et al. 2004), mice (McRae et al. 2005) and humans (Engmann et al. 2006). PGRMC2 protein has a high homology to PGRMC1 and has been identified in reproductive tissues of cows (Kowalik et al. 2013b, Saint-Dizier et al. 2012, Slonina et al. 2012), monkeys (Keator et al. 2012) and mice (Zhang et al. 2008). In our study, *PGRMC1* mRNA was present in the CL of the domestic cat, with highest amounts during the pre-implantation period of pregnancy, but with no changes throughout the non-pregnant luteal phase. In contrast to this, *PGRMC2* expression remained unchanged in CL of pregnancy, but increased in CL of early regression compared to formation in the non-pregnant luteal phase. While the role *PGRMC1* plays during the pre-implantation period might be similar to that discussed above for *PGR*, the contribution of *PGRMC2* in the feline luteal phase is uncertain. No clear physiological action is yet known for *PGRMC2*, although its involvement in progesterone signaling and cytochrome P450 binding is under discussion (Wendler and Wehling 2013).

Estrogen and its receptors are well known to play critical roles in the female reproductive cycle and are involved in the maintenance of pregnancy. The actions of estrogen in the ovary are diverse and many of them are implemented *via* nuclear receptors (Rosenfeld et al. 2001). Mouse models with knock-out of the estrogen nuclear receptor are infertile, emphasizing the essential role of estrogen receptivity in the reproductive system (Lubahn et al. 1993). Expression of ESR1 and ESR2 has been shown in CL of rats (Telleria et al. 1998), monkeys (Duffy et al. 2000), humans (Iwai et al. 1990) and sheep (Ott et al. 1993). In addition, several *ESR1* splicing variants have been described in feline tissues, and their potential regulatory role in the responsiveness of gonadal cells to hormone stimuli was proposed (Cardazzo et al. 2005,

Schoen et al. 2012). In our study, *ESR1* expression increased significantly with progression of the domestic cat non-pregnant luteal phase and showed a tendency to increase during regression in pregnancy as well, while no changes were observed for *ESR2* expression. These results suggest that the estrogen signal in the domestic cat CL is regulated mainly *via ESR1* and might play a role in luteal regression. The source of estrogens could be of extraluteal origin, since the capacity of the analyzed cat CL to produce estrogen and the intraluteal estrogen concentrations decrease with the luteal regression (Amelkina et al. 2015, Zschockelt et al. 2014). In the Iberian lynx, *ESR1* expression was higher in perCL compared to frCL, which was reversed for *ESR2* expression. Such reversed expressions are discussed in the ovary for ESR2 modulation of ESR1-mediated gene transcription and referred to as "Ying Yang" relationships in mice (Bottner et al. 2014, Lindberg et al. 2003).

Apart from its nuclear receptors, estrogen can also transduce its actions *via* the non-genomic pathway (Soltysik and Czekaj 2013). One of the components of this rapid pathway was recently identified as GPER1, whose actions in reproductive tissues are still being investigated (Mizukami 2010, Prossnitz and Maggiolini 2009, Thomas et al. 2010). In mice, GPER1 has been identified in CL and was suggested to mediate the stimulating effect of estrogens on progesterone synthesis (Liu et al. 2015). The expression of *GPER1* in CL of Iberian lynx replicated the *ESR1* profile and was higher in perCL compared to frCL. This may indicate a coupled or parallel action of ESR1 and GPER1 in lynx CL. In contrast, no changes in GPER1 expression were observed throughout the luteal phase of domestic cats, suggesting a minor regulatory role of this receptor in formation, maintenance and regression of the domestic cat CL.

The critical involvement of androgens in the ovarian cycle has been demonstrated on AR-knockout mouse models, which were infertile mainly due to dysfunctional ovulation (Cheng et al. 2013). This, together with further studies on androgen actions, showed that androgens themselves can implement an effect *via* AR signaling and not only by serving as a substrate for

estrogen synthesis. Similarly, androgens were shown to stimulate progesterone production and reduce numbers of apoptotic cells in rat CL, thus exhibiting luteotropic characteristics (Carrizo et al. 1994, Goyeneche et al. 2002, Takiguchi et al. 2000). This is interesting because mRNA amounts of *AR* in our study were higher in perCL compared to frCL of the Iberian lynx. Based on the luteotropic actions of androgens *via* AR in the rat CL, we may consider similar contributions of AR to the perCL of lynx. In contrast to this, the expression of AR increased with the regression of CL in domestic cats. The role of AR in the luteal phase of the domestic cat is thus unclear. Expression of AR is also found in the ovary of rats (Szoltys et al. 2007), pigs (Slomczynska et al. 2006) and humans (Horie et al. 1992).

In summary, we identified mRNA of *PGR*, *PGRMC1*, *PGRMC2*, *ESR1*, *ESR2*, *GPER1* and *AR* in CL tissue of domestic cats and lynx, showing that the feline CL is theoretically receptive to sex steroid hormones throughout its life span. Changes in mRNA amounts of these receptors throughout the domestic cat luteal phase may indicate their involvement in the formation, maintenance and regression of the feline CL. Moreover, high mRNA amounts of *PGRMC1*, *PGRMC2*, *ESR1*, *GPER1* and *AR* in perCL of the Iberian lynx suggest that the physiological persistence of lynx CL might be partly mediated by steroid action on this gland. Detection of PGRMC1 protein in frCL and perCL of the Iberian lynx might suggest that the action of progesterone in lynx can be primarily regulated via this membrane receptor. Our results contribute to increasing knowledge about the feline luteal cycle and provide some insight into possible molecular pathways of steroid actions in physiological persistence of the CL. Together with our findings, future research on luteotropic and luteolytic factors in the CL will broaden our knowledge on wild felid reproduction and possibly assist in developing reproductive techniques for endangered feline species, which, predominantly include the Iberian lynx.

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5 GENERAL DISCUSSION

5.1 Corpus luteum of the domestic cat

Previously, there have been histological descriptions of the domestic cat luteal phase that were limited to pregnancy (Dawson 1941) and its early stages (Roth et al. 1995). In current work, it is clear that the domestic cat CL of pregnancy and non-pregnant luteal phases go through the same stages of formation, development/maintenance, early and late regression. However, the rate of these changes in the non-pregnant luteal phase is unknown due to the blind sample acquisition, i.e., no information on the day of ovulation in cats. The observed histomorphological characteristics reflected functional state of the CL, as evidences by relation of development/maintenance stage with the highest intraluteal P4. It is worth noting, that the CL found in a state of structural regression still produced measurable amounts of P4, although overall concentration significantly decreased from development/maintenance to early regression stages. This indicates on somewhat intertwined functional and structural luteolysis, where the structurally regressing CL is still capable of producing P4. Overall, described histomorphological markers are applicable for staging of the CL and, as will be discussed below, were used in characterization and state identification of the lynx CL.

Measured intraluteal P4 and estrogens followed the typical serum profile reported before in the domestic cat (see Section 1.5 for a short summary), except for the absence of estrogen surge prior to parturition. This may either indicate the consequences of an incomplete sampling for pregnancy (absence of days later than 45) or on an extraluteal source of this surge. The latter is much more probable, as the waves of follicle development are discussed to surface during the luteal phase in the domestic cat and are a potential source of rising estradiol (Shille et al. 1979, Wildt et al. 1981). Moreover, subsequent study on steroidogenic capacity of the same domestic cat CL with additional day 63 samples revealed no elevation in aromatase expression (Zschockelt et al. 2014).

In their study on serum P4 profiles in the domestic cats, Verhage with colleagues were puzzled by the elevated P4 peaks in pregnancy compared to pseudopregnancy and stated that this "..is an endocrinological problem of considerable interest, worthy of additional study" (Verhage et al. 1976). Unfortunately, results of current study cannot solve this problem, as it is not possible to compare the intraluteal peaks of P4 between pregnancy and the non-pregnant luteal phase due to the low sample size. However, the difference in P4 production by the CL itself can still be the cause of these distinctions in peaks of serum P4, particularly because the placenta contribution is not mirrored in peripheral P4 profile. If it is so, then the differentiation between pregnant and non-pregnant luteal phases and thus the decision on the CL life span and function can already be present by the time of P4 peak levels, the time-point of which varies greatly between individuals, ranging from days 11 to 30 (Verstegen et al. 1993).

The domestic cat CL proved to express nuclear and membrane receptors to steroids at all stages of its development and regression. This is in contrast to some other species like rats, whose ovary expresses PGR only for a short time during ovulation, and no PGR can later be detected in luteal cells (Park and Mayo 1991), and primates, whose CL show prevalence towards ESR2 expression (Duffy et al. 2000, Hild-Petito and Fazleabas 1997). Significant changes in the expression of at least one type of receptors for P4, estrogens and androgens throughout the luteal phase suggest a regulation of the domestic cat CL by these steroid hormones. It is possible that, similarly to dogs (Hoffmann et al. 2004), P4 plays a role in the formation of the feline CL, as well as exhibits paracrine/autocrine stimulation of initial CL secretory activity, transducing its signal *via* PGR and/or PGRMC1.

The effect of estrogens on the non-pregnant luteal phase in the domestic cat is unclear. The rise in ESR1 expression may potentially result in the increased receptivity of the CL to estrogens towards the end of luteal phase, which in turn might coincide with the observed rise in serum estradiol (Verhage et al. 1976). The lack of information on ovulation date in analyzed samples from non-pregnant domestic cats prevents the timing of intraluteal ESR1 rise with

reported in the literature estradiol surges. If elevated estradiol at the end of luteal phase indeed acts on the regressing CL, there might be a possibility of its luteolytic action. In cows, estradiol can initiate luteolysis and is proposed to contribute to the control of the CL life span (Salfen et al. 1999). However, studies on the change in intraluteal receptor expression throughout the bovine luteal phase indicate on the possible role of ESR1 in luteal maintenance, while ESR2 is associated more with the CL regression (Amrozi et al. 2004, Berisha et al. 2002, Shibaya et al. 2007). Estrogens are known to be luteolytic in primates, although this action seems to be transduced rather *via* ESR2, who in contrast to ESR1 is present in the CL on protein level and can activate ESR1 pathways (Duffy et al. 2000). From existing literature on the domestic cat it is not clear what effects do estrogens have on the CL and whether they are mainly luteotropic or luteolytic. Clearly, subsequent functional studies on protein level have to be performed to advance us in understanding of estrogen role in the feline CL.

Similarly to discussed above ESR1, AR significant increase with the regression of the CL raises multiple questions with no clear answer. In rodents, androgens act luteotropic and have been shown to secure the rat CL from apoptosis (Goyeneche et al. 2002, Takiguchi et al. 2000, Thordarson et al. 1997). The potential raise in receptivity to androgens of the domestic cat CL with the start of structural regression may propose an involvement of androgens in luteolysis. Another hypothesis is that during this AR rise, androgens are not available to the CL and thus cannot prevent even highly receptive CL from the regression. However, analysis of the same CL from domestic cats has revealed the presence of intraluteal androgens also during stages of regression (Zschockelt et al. 2014).

In regards to this part of the study, it is important to remember the high complexity of molecular pathways of the steroid action. The initial signal of a ligand can be altered at the stage of the transcription mediation *via* co-activators and co-inhibitors, and, moreover, there can still exist yet uncharacterized receptors and their isoforms (Jamnongjit and Hammes 2006, O'Malley

2005). The research is thus highly challenging, but every new step in unraveling each steroid action and its pathway, particularly in the CL, is of a great value.

It has been shown in this work that apart from steroid hormones, the domestic cat CL falls under the regulation of some of the apoptosis-related factors. Elevated in both preimplantation period of pregnancy and formation stage of the non-pregnant luteal phase, TNFRSF1A may contribute to the processes of tissue remodeling during luteal formation in the domestic cat. In both rat and bovine CL, TNFRSF1A has been tightly associated with the processes of luteolysis (Abdo et al. 2008, Hojo et al. 2010). Pro-apoptotic factor FAS is a known player in regulation of the luteal regression in many species (Galvao et al. 2010, Kuranaga et al. 2000, Taniguchi et al. 2002); this factor, however, seems to have a less prominent role in the domestic cat luteal phase (based on mRNA levels). Only during pregnancy, expression of FAS was significantly higher in formation and regression stages of the CL. On the contrary, the expression of a known anti-apoptotic factor TNFRSF1B increased with the start of the CL regression. This is even more perplexing, as TNFRSF1B does not possess the death domain and, moreover, has been shown to activate nuclear factor-κB, which is involved in cell survival and protection from apoptosis (Shishodia and Aggarwal 2002, Thommesen and Laegreid 2005). The role of TNFRSF1B in the domestic cat regression stage is thus unclear.

5.2 Corpus luteum of the lynx

Based on the established histomorphological classification of the domestic cat luteal phase, it was possible to stage the obtained CL as early regression (perCL of Eurasian lynx before mating), development/maintenance (perCL of Iberian lynx after mating) and formation (frCL of Iberian lynx). The first observation that can be made is the structural difference between perCL of Eurasian and Iberian lynx. While perCL of Eurasian lynx showed signs of structural regression, perCL of Iberian lynx contained mostly intact cells with characteristics of a functional maintained feline CL. These differences might be induced by the presence of freshly

ovulated CL together with perCL in Iberian lynx, rather than by species differences. This observation suggests that the structural and possibly functional state of the perCL changes under the influence of a new ovulation, returning it to a state of maintenance. One of the contributors to this structural rescue can be P4, which could transduce its pro-survival action *via* PGRMC1 and potentially PGRMC2 in perCL. Another possible tropic and anti-apoptotic support may come from androgens *via* AR. These factors, together with anti-apoptotic TNFRSF1B, may play a significant role in the perCL revival and transition from regression stage to its maintenance. The action of estrogens in perCL is not clear, as ESR1 and ESR2 exhibit reverse patterns. It might be possible that, as in bovine CL (Amrozi et al. 2004, Berisha et al. 2002, Shibaya et al. 2007), estrogens transduce different effects *via* each receptor, e.g., maintenance of perCL *via* ESR1, but not ESR2. The analysis of intraluteal estrogens indicated a tendency of higher levels in perCL of the Iberian lynx after mating, further supporting the potential involvement of estrogens in perCL maintenance. Cooperation between ESR1 and GPER1 has been reported in some cells, e.g., human ovarian cancer cells (Albanito et al. 2007), and promoted cell division, while silencing of any one of them inhibited this effect (Soltysik and Czekaj 2013). Possible parallel- or co-action of ESR1 and GPER1 may exist in the lynx CL, promoting the integrity and/or function of perCL.

5.3 Luteal life span in the feline species

The actual structural life span of the domestic cat CL is somewhat not well documented. In feline literature, the CL is termed as regressed when serum P4 levels fall to nadir (< 1 ng/ml). At the same time, it is evident, particularly in this study, that CL can sometimes still be present in the ovary throughout anestrus and in subsequent diestrus in domestic cats. However, such CL of previous cycles showed histomorphological composition of *corpus albicans* and their intraluteal P4 content was almost undetectable. During lactation, CL can remain in the ovary and even exhibit partial structural integrity for at least 63 days after parturition, as documented

by Dawson (1946) and this study. So far, there are no reports on the domestic cat CL exceeding this life span. In contrast, CL of Eurasian lynx appear to have a life span of at least two years, or at least three ovarian cycles. The CL of bobcats are thought to never actually disappear from the ovary, and Crowe (1975) in his study counted as many as 59 CL in one old female bobcat. The differences between these reports on the bobcat and Eurasian lynx is that perCL of the latter still exhibited functional activity, while perCL of bobcats are termed in some literature as CAs. On the other hand, histologic appearance of such perCL in the study of Duke (1949) didn't reflect that of a typical CAs, as they still contained population of intact luteal cells in September, October and even November. This functional appearance of perCL in bobcats may be related to the incidences of estrus outside the defined breeding season, and thus the influence of new ovulations on the present perCL. Such influence can be supported by studies on the luteal cells derived from perCL of bobcats and their treatment with human chorionic gonadotropin (hCG), which resulted in the increase of P4 production. It is worth mentioning, however, that animals in Duke's study were from southern Colorado and New Mexico and, therefore, exhibited relatively short breeding season that could be comparable to that of Mississippi bobcats (February – March; Stys and Leopold 1993). The chance of incidental ovulations during late autumn in these animals thus remains low, what might propose partial functionality of bobcat CL for some period after parturition.

5.4 Luteolysis in the feline species

In regardless of its actual structural life span, the CL of domestic cats undergoes an evident regression till the scar-like condition of the CA by the end of each luteal phase. The process of luteolysis and differences in it between pregnant and non-pregnant luteal phases in the domestic cat is not yet known. There are several scenarios for the termination of CL life span in mammalian species. For instance, in dogs, no active luteolytic signals are evident during pseudopregnancy, as contrary to prepartum luteolysis *via* $PGF_{2\alpha}$ mechanism, resulting in a

passive regression and prolonged life of the CL (Kowalewski 2014). On the other hand, in ruminants, CL of infertile cycles undergo active, mainly $PGF_{2\alpha}$ induced, luteolysis to enable initiation of a new ovarian cycle; while CL of fertile mating are rescued from regression by subversions of this uterine luteolytic signal (McCracken et al. 1999). In rodents, CL of spontaneous cycles never develop to their full functional state, thus not exhibiting gonadotropin inhibition and allowing follicular development and ovulation within a few days (Smith et al. 1975). Fully functional CL that develop after infertile mating undergo rapid functional luteolysis partly *via* $PGF_{2\alpha}$ induced expression of 20α-hydroxysteroid dehydrogenase, which metabolizes P4 to 20α-dihydroprogesterone and thus allows the female rat to recycle while CL are still structurally present in the ovary (Stocco et al. 2007, Stocco et al. 2000).

The non-pregnant luteal phase of the domestic cat might be either actively terminated by luteolytic agents or have a limited time span due to the absence of support from luteotropic factors. The second scenario might explain the increase in expression of potential luteotropic factors (e.g., AR, TNFRSF1B) during late non-pregnant luteal phase: the CL still produces receptors potent for survival likewise in pregnancy, but either lacks the activation of the survival cascade by ligands or the tropic signal is blocked on a downstream level. This may also explain high variations in reported functional life spans of the CL in non-pregnant luteal phases in domestic cats, e.g., from 42 to 62 days as evidenced by serum P4 levels (Verhage et al. 1976, Wildt et al. 1981). If the CL of a non-pregnant luteal phase is not limited in its functional and structural life by timely luteolytic signals, it may survive in the ovary for various lengths, undergoing passive regression, similarly but shorter as in dogs (Kowalewski 2014). The CL of pregnancy then would survive longer, being rescued by luteotropic factors from the mid of luteal phase, which may include ESR1 (if estrogens are luteotropic in domestic cats), AR and TNFRSF1B. Such luteotropic support may also come from prolactin, as structurally intact CL of lactation were reported to remain in the ovary after parturition (Dawson 1946).

Discussion is more complicated for the lynx, as in this species CL seem to not undergo complete luteolysis at any stage. It is possible that perCL start to regress slowly after parturition and weaning, resulting in the observed stage of regression in Eurasian lynx before the next estrus. However, with the new ovulation and under possible influence of frCL and/or gonadotropin surge, these perCL return to the state of structural maintenance. This is interesting, as based on the results of this work, perCL of lynx are prepared for initiation of apoptosis, as evidenced by relatively high mRNA amounts of *CASP3* and increased expression of pro-apoptotic factor *FAS*. And yet, no structural regression was observed in perCL of Iberian lynx and no positive staining for activated CASP3 protein could be detected. There might be at least three possible explanations for this phenomenon: (i) the absence of luteolytic signal and, therefore, no activation of the apoptotic cascade, (ii) blockage of the transducing luteolytic signal on the downstream of the apoptotic cascade and, naturally, (iii) lack of the clear information on protein function and additional factors that may change the initial picture. The first hypothesis might not be completely valid, as it has been shown that metabolite of commonly luteolytic $PGF_{2\alpha}$ (PGFM) increases in the urine of Iberian lynx towards the end of pregnancy (Finkenwirth et al. 2010). However, CL survive this luteolytic signal and transition into perCL after parturition. Here, $PGF_{2\alpha}$ might play a role in promoting luteal regression of new CL at the end of pregnancy (Zschockelt et al. unpublished results). The second hypothesis can be supported by current results, i.e., increase in the expression of pro-survival factors *TNFRSF1B* and *BCL2* in perCL. Working on the downstream of apoptotic cascade, BCL2 is able to block the initiated apoptotic signal on the inner cell level, by preventing the activation of BAX and, therefore, the permeability of mitochondria. Moreover, P4 has been shown to promote BCL2 expression and decrease BAX to BCL2 ratio in bovine luteal cells (Liszewska et al. 2005); P4 of perCL can, therefore, play a protective role *via* this mechanism as well. TNFRSF1B may compete for the ligand TNF binding with TNFRSF1A, resulting in the activation of pro-survival rather than pro-apoptotic pathways. The possible inhibition of

FAS/Fas ligand signal might occur already on caspase-8 level. Another possibility is that FAS may rather act anti-apoptotic in the lynx perCL, as has been concluded for some cells (Peter et al. 2007) and in one study for the bovine CL (Duncan et al. 2012).

5.5 Physiological persistence of the corpus luteum

The phenomenon of physiological CL persistence is not common among mammalian species. So far, Eurasian, Iberian and Canada lynx are the only species where both structural and functional physiological persistence of CL has been reported. In cows, the persistence of such functional CL is considered pathological, as it prevents the occurrence of a new ovarian cycle (Magata et al. 2012). It is unclear whether perCL in bobcats are functional, however, it is suggested that structurally these perCL might remain in the ovary for several years (Crowe 1975) and be still responsive to gonadotropin treatment for at least two breeding seasons (Woshner et al. 2001). There are only three more reported examples of a prolonged structural CL life span in different species. The Scottish wild cat (*Felis silvestris grampia*) was documented to retain slowly regressing CL for many months after parturition, but with no reports on CL presence during subsequent ovarian cycles (Matthews 1941). While in the *Cetacea* literature, persistence of CL throughout the lifetime of an animal is a common observation (Hirose et al. 1970, Marsh and Kasuya 1984, Ohsumi 1964). These perCL in *Cetacea* species are termed CAs, however, histological studies on Short-Finned Pilot Whale (*Globicephala macrorhynchus*) and Blue white dolphin (*Stenella caeruloalba*) indicated the presence of intact luteal cells in analyzed CAs (Hirose et al. 1970, Marsh and Kasuya 1984); moreover, authors start applying the term “young CA” the moment the CL enters regression stage. The same terminology difference exists in the literature on spotted hyenas (*Crocuta crocuta*), where probably still functioning but regressing CL are already termed CAs, and were argued to persist in the ovary for years (Matthews 1939). Unfortunately, no hormone

measurements have been performed in perCL of these species and it is thus unknown whether they retain their functionality, i.e., P4 production.

Advantages of the CL physiological persistence have also been discussed in the literature. Observing the persistence of CL from previous cycles in bobcats, Crowe (Crowe 1975) speculated that these perCL might still secrete P4 in conjunction with CL of a current pregnancy. Subsequently, Woshner and colleagues (Woshner et al. 2001) tested this assumption by measuring intraluteal P4 and stimulating luteal cells of perCL with hGC. The sample size was insufficient for drawing a conclusion of functional state of perCL; however, derived from them luteal cells were responsive to hGC treatment and exhibited elevations in P4 secretion. This further led to the discussion that perCL of previous cycles may contribute to the maintenance of early pregnancy by reinitiating progesterone secretion, or aiding in the regulation of gonadotropin secretion (Woshner et al. 2001). In hyenas, the function of perCL is also discussed, and their contribution to the protection of genitalia from the action of estrone by their P4 secretion is suggested (Matthews 1939).

Functional persistence of CL in Eurasian, Iberian and Canada lynx has been hypothesized to secure monoestrous cycles in these species (Fanson et al. 2010, Goritz et al. 2009, Painer et al. 2014). Indeed, P4 is a known suppressor of ovarian activity (Croxatto 2002), and priming with progestins has been used in domestic cats to prevent spontaneous ovulations prior artificial ovulation induction with equine and human CG (Stewart et al. 2010). Subsequent studies also revealed that such progestin priming increased ovarian sensitivity to gonadotropin stimulation and could partially mitigate the anomalies caused by artificial induction (Stewart et al. 2012). It is tempting to speculate that monoestrous lynx might have improved rates of their ovulations due to the natural progestin priming of perCL by requiring smaller gonadotropin surges. However, there is no information on sensitivity of lynx ovaries to exogenous gonadotropins, while this sensitivity may vary widely across felids (Pelican et al. 2006), and there are no studies on how such priming can affect ovarian environment in naturally ovulating individuals.

Moreover, progestin priming results in decreased levels of estradiol, while lynx are evident to produce estrogens in the presence of functioning perCL (this work and Zschockelt et al. 2015). Finally, perCL may contribute to the support of a new pregnancy, as suggested for bobcats (Woshner et al. 2001), either by sole P4 production or with expression of additional luteotropic factors. In horses and elephants, the role of such pregnancy support falls on accessory CL (Allen 2001, Hildebrandt et al. 2011), although they haven't been reported to persist in the ovary till the next cycle.

The results of this study introduce potential mechanisms of CL persistence in the Iberian lynx. Androgens may secure lynx perCL from apoptosis and stimulate P4 production *via* AR, as such action is also evidenced in rat CL (Goyeneche et al. 2002). The rescue of perCL from regression stage may be implemented by P4 of either frCL or perCL origin, which is also in concordance with studies on rats, where P4 is claimed a direct factor for CL survival (Goyeneche et al. 2003). In the lynx perCL, supporting action of P4 seems to be mainly regulated by PGRMC1 and potentially PGRMC2, rather than PGR. The action of estrogens might be beneficial for perCL survival in lynx, e.c., they may stimulate expression of P4 receptors (Horwitz et al. 1978), thus supporting P4 rescue pathway. Finally, anti-apoptotic factors TNFRSF1B and BCL2 might be involved in structural integrity of perCL, and P4 might potentially stimulate BCL2 expression.

It is clear that other factors, which were not investigated in this work, can play a critical role in the persistence of lynx CL. For instance, prostaglandin E_2 (PGE_2) is a potential luteotropic factor in felids and a high expression of its synthase and receptor has been shown in perCL of Iberian lynx (Zschockelt et al. unpublished results). Additionally, preliminary results of our group indicate on the involvement of prolactin and LH in the support of the CL life span, what would be in accordance with luteotropic role reported for these factors in other species (Bachelot and Binart 2007, Chen et al. 2002, Hoffmann et al. 2004, Niswender et al. 2000).

The distinction of monoestrous Eurasian, Iberian and Canada lynx from polyestrous bobcats, as well as from other mainly polyestrous felids, raises a question of the CL evolution in these

species. One hypothesis is that fully functional perCL arose in these lynx species after their divergence from the bobcat lineage, which is the oldest species of modern *Lynx* genus (Johnson et al. 2006). This would also partly explain why no other felid studied so far has a similar mechanism of physiological CL persistence. The conditions for developing of functional perCL would have already been present in bobcats, as their CL structurally persist in the ovary, possibly due to the extremely low rate of regression (Crowe 1975), and are responsive to gonadotropin stimulation (Woshner et al. 2001). The harsh environmental conditions in which Eurasian, Iberian and Canada lynx found themselves, i.e., winter seasons in Canada and Northern Europe and dry seasons in Iberian Peninsula, might have promoted the transition of only structurally perCL into fully functional ones to turn the initially polyestrous cycle into monoestrous and thus, e.g., secure the birth and weaning of cubs during the most favorable time of the year.

5.6 Concluding remarks

The results of current work shed some light on the processes of CL formation, maintenance and regression in the domestic cat. Changes in CL structure, as well as in expression levels of potential luteotropic and luteolytic factors throughout pregnancy and the non-pregnant luteal phase are documented. Moreover, some of the structural and endocrine events during perCL life span in Iberian and Eurasian lynx are reported, and potential mechanisms of physiological persistence involving factors studied are discussed. This study is an important basis for any further research on reproduction in the domestic cat and wild felids, particularly critically endangered Iberian lynx. The comparison between domestic cat and lynx species has to be performed very carefully, and it is already evidenced that these two felids differ remarkably in some of their mechanisms of luteal function.

The CL gland and its P4 function has only recently been discovered, and there is still a high number of studies necessary to be performed for advancing us in our understanding of luteal

function and its contribution to the female reproduction patterns, thus to the species survival. High variations in luteal phase across the species and particularly inside the *Felidae* family, i.e., studied here domestic cat and lynx, prompts us for a highly species-specific investigation approach and further supports Duke's remark (1949):

"With the *corpus luteum*, as with other physiological and histological aspects of the mammalian reproductive tract, the urge to make generalizations is tempting, but the pitfalls are numerous"

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Selbständigkeitserklärung

Ich erkläre ausdrücklich, dass es sich bei der von mir eingereichten schriftlichen Dissertation um eine von mir selbstständig und ohne fremde Hilfe verfasste Arbeit handelt. Den Anteil der Koautoren an den gemeinsamen Publikationen habe ich kenntlich gemacht.

Die Dissertation wurde bisher nicht für eine Prüfung oder Promotion oder ähnlichen Zweck zur Beurteilung eingereicht.

Ich erkläre ausdrücklich, dass ich sämtliche in der Dissertation verwendeten fremden Quellen, auch aus dem Internet als solche kenntlich gemacht habe.

Ich erkläre ausdrücklich, dass ich mich über die Promotion-Regelungen informiert habe.

Berlin, den…..

Olga Amelkina

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